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STUDIES on PEPTIDASES

by

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Submitted in partial fulfilment
of the requirements for the degree
of Doctor of Philosophy at the
University of Warwick.

DEPARTMENT OF CHEMISTRY

MARCH 1990

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Abbreviations

BSA	Bovine serum albumin
HSA	Human serum albumin
DAPI/IV	Dipeptidylaminopeptidase I/IV
AcAP	Acetylaminopeptidase
DEAE	Diethylaminoethyl
h	hour(s)
min	minute(s)
tris	Tris(hydroxymethyl)aminoethane
sec	second(s)
U	Units
mg	milligram
HPLC	High Performance/Pressure Liquid Chromatography
PAGE	Polyacrylamide gel electrophoresis
IEF	Isoelectric Focussing
IEP	Isoelectric Point
FABMS	Fast Atom Bombardment Mass Spectroscopy
C-term	Carboxy terminal/us
N-term	Amino terminal/us
AANA	Acetyl-L-Alanyl-p-Nitroanilide
MEP	Mercaptoethanol / EDTA / Phosphate Buffer
S/N	Supernatant

Unless otherwise stated a Unit of enzyme is that amount of enzyme which will hydrolyze one micromole of substrate per minute per mg of protein under stated conditions.

$$1U = \mu\text{moles/mg/min}$$

DECLARATION

The work described in this thesis is the original work of the author except where acknowledgement is made to work and ideas previously published. It was carried out in the Department of Chemistry, University of Warwick, between October 1985 and September 1988, and has not been submitted previously for a degree at any Institution.

Publication

"The Preparation and Properties of Immobilized Dipeptidyl Aminopeptidase (DAPI, Cathepsin C, E.C.3.4.14 .1)".

Hutchinson, D.W. and Tunncliffe, A. (1987) *Biochemica et Biophysica*

Acta 916, 1-8.

ACKNOWLEDGEMENTS

I would like to thank the Chairman of the Department of Chemistry, Professor M.G.H. Wallbridge for the use of Department facilities.

I am indebted to Professor K.R. Jennings, Dr Adrian Woolfit and Dr David Mitchell for valuable discussions pertaining to my research. I would also like to thank members of the Department, and in particular C421, both past and present for the convivial and unique atmosphere in which to work.

Above all I would like to thank my supervisor, Dr D.W. Hutchinson for his unrelenting encouragement and advice during the course of this work.

Abstract

This study details the methods of purification of the peptidase enzyme, Diaminopeptidase I (DAPI, Cathepsin C) and an acetyl-amino acid releasing enzyme called Acetylaminopeptidase (AcAP). The preparations obtained were fully characterized and identified as being homogenous preparations of the above enzymes. Together with Diaminopeptidase IV (DAPIV) it is intended to use these enzymes to hydrolyse unknown derivatized peptides and proteins for sequencing studies.

Several different techniques were used in my studies. They included three different methods for the estimation of molecular weights, two methods for the determination of isoelectric points, an activity stain, inhibition studies, substrate specificity, studies and storage studies.

Chapter 3 details the use of DAPI in an immobilized form to hydrolyze a peptide, with subsequent separation of the digestion products with sequence analysis by HPLC. This work culminated in a publication (Ref70).

Many results confirm those found for the enzymes by previous workers. However, the metal ion inhibition studies of AcAP, in particular, contains some results contrary to some published results but possible reasons for this are offered.

In summary, this study details methods for the purification of two enzymes postulated to be of use in the sequencing of some derivatized peptides. Further work, which takes the form of the use of the enzyme

cocktail was carried out but these studies were incomplete and therefore not included in this study. However the techniques which would have been used and the conditions which would have been employed have been included in the introduction for the sake of completeness.

Chapter 1

Introduction

1.1 Experimental Basis

1.1.1 Reasons for Study

Little is known about the structures and properties of plant proteins when compared to the vast knowledge compiled for animal proteins. In recent times, further characterization of some plant proteins i. e. legumes has become much more important because they are frequently used as food additives. Structural differences in the plant proteins can have effects upon their suitability for addition to foodstuffs. Hence, the development of a rapid sequencing technique, which would supercede the existing techniques by its simplicity, speed of use and wider-ranging application is required. It is hoped that this study will provide some information towards the realization of just such a technique. (Ref. 1)

Classical methods (section 1.1.2) are still very much a part of sequencing studies, but they tend to become of little use when faced with a large number of proteins which are amino-terminally blocked by acetylation (eg. Ovalbumin, Ac-Gly-Ser-Ile-Gly-Ala) or formylation, or other functional groups as a result, in cases, of post-transcriptional modification. (Ref. 2)

Furthermore, it has proved desirable to develop a method which reduces the chemical treatment of a sample, thereby minimizing artefactual results, and which can be applied to many varied samples.

1.1.2 Established Methods of Protein Sequencing

There are several methods already established for the sequencing of proteins. Many involve the sequential removal of each amino acid in the polypeptide chain by enzymic or chemical means with subsequent identification of the cleaved residue. A simple method is based on the sequential hydrolysis of amino acids from the carboxyl terminus of a polypeptide with identification of the resultant products by chromatography (eg. paper or 'soft'-column) against standard amino acids. The accuracy of this method lies in the frequency with which the samples of a polypeptide digest eg. with Carboxy peptidase A are removed, and upon the resolution of the digestion products. A further drawback is that the enzyme, after removal of one carboxy-terminal amino acid proceeds to remove another residue. Therefore one must monitor the rate of liberation of residues which will be directly related to the type of amino acid at the terminus.

Established methods are based upon the sequential derivation or cleavage of amino acid residues from the carboxyl-, amino-terminals or by selective hydrolysis of the polypeptide into smaller fragments.

Identification of the carboxyl-terminal amino acid of peptides can also be performed by several chemical means. For example, the C-terminal amino acid can be reduced to the corresponding α -amino alcohol. Upon complete hydrolysis, the hydrolysate will then contain one molecule of an α -amino alcohol which can be identified quite easily by chromatography, thus identifying the C-terminal residue. Hydrazinolysis (Fig. 1.1.2 (a)) cleaves all the peptide bonds by converting all except

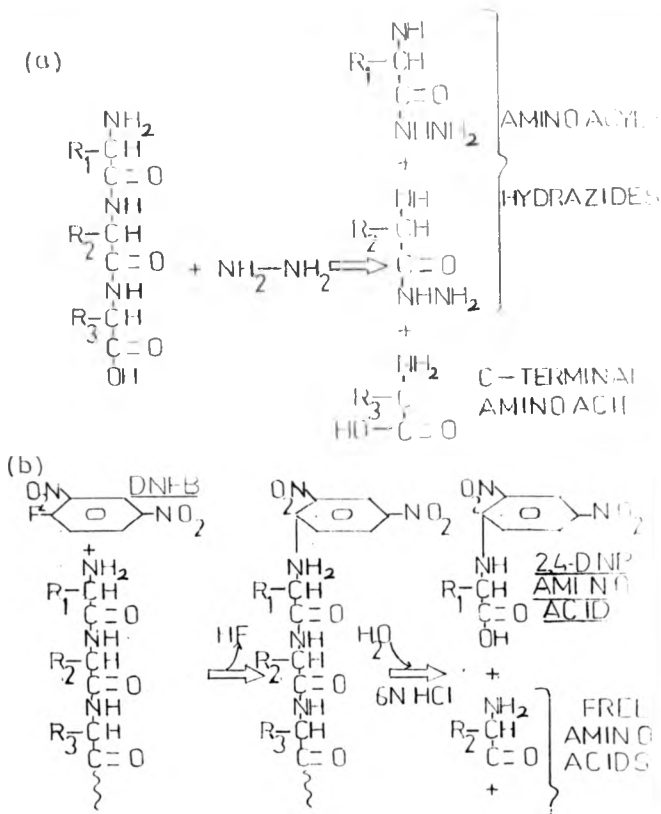


Fig. 1.1.2 (a) Identification of the C-terminal Residue by Reaction with Hydrazine (Hydrazinolysis) (Ref.3)

Fig. 1.1.2 (b) Identification of the N-terminal Residue of a Peptide by the Sanger Reaction (Ref.3)

the C-terminal residue into hydrazides. The reaction mixture then only contains one native amino acid, which could be identified by chromatographic analysis. (Ref.3)

The most common approach to amino acid sequence analysis utilizes the identification of each N-terminal amino acid moiety in successive 'rounds' of derivatization, hydrolysis, separation of derivatized residues and the unreacted peptide chain, followed by chromatographic identification. A classic method, and one of the first to be employed was developed by Sanger (Fig. 1.1.2 (b)), who found that the free unprotonated α -amino group of a peptide reacts with 2,4-dinitrofluorobenzene (DNFB) to form the yellow 2,4-dinitrophenyl-amino-acid derivatives. Having obtained such a peptidyl derivative, all of the peptide bonds are cleaved by hydrolysis with 6N HCL (which leaves the bond between the α -amino acid and 2,4-dinitrophenyl group intact). Consequently, as with hydrazinolysis, the hydrolysate contains only one derivatized amino acid unique in the mixture. The labelled N-terminal residue can easily be separated from the mixture of unsubstituted amino acids and identified by chromatographic comparison to DNP-derivatized amino acids. Of course, if one were to use this method for sequencing studies, one would require prohibitive amounts of peptide sample, even if one employs the chemical labelling moiety, 1-dimethylaminonaphthalene-5-sulphonyl chloride (dansyl chloride), which has been found to be 100x more sensitive than Sanger's reagent method in preparing a derivatized peptide. This method ultimately uses a large amount of sample because each 'round' terminates in full hydrolysis of the peptide which has been derivatized (Fig. 1.1.2 (c)). (Ref. 4)

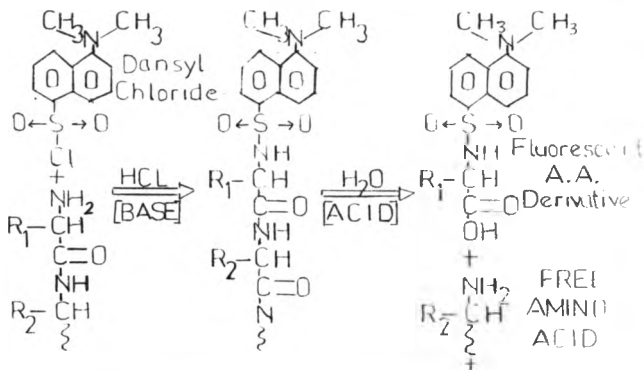


Fig. 1.1.2.(c) Identification of the N-terminal Residue of a Peptide by Reaction with Dansyl Chloride (Ref. 3)

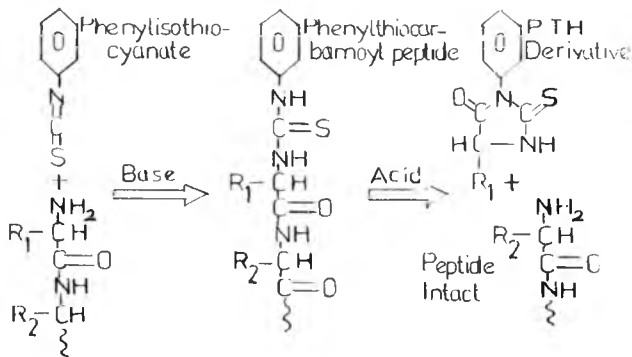


Fig. 1.1.2.(d) Identification of the N-terminal Residue of a Peptide by the Edman Degradation Method (Ref. 3)

The most widely used method today overcomes the need for large amounts of sample, because repeated 'rounds' of this procedure can be conducted on the same peptide. The Edman degradation of peptides uses phenylisothiocyanate which reacts quantitatively with the free amino group of a peptide to yield the corresponding phenylthiocarbamoyl peptide. Treatment with anhydrous acid cleaves the N-terminal derivatized amino acid, but leaves the rest of the peptide chain intact. The phenylthiocarbamoyl amino acid is then cyclized to its phenylthiohydantoin derivative which is readily analyzed and identified by gas-liquid chromatography (Fig. 1.1.2 (d)). (Ref. 3)

As this procedure allows for successive cycles on the same peptide, this technique is amenable, and has been used for an automated amino-acid "sequencer", perfected by Edman and G.Begg. (Ref. 6)

However, some native proteins have their N-terminus buried deep within themselves or are derivatized e. g. by acetylation, which sterically hinders the labelling techniques. Therefore, other techniques which may entail partial denaturation or selective cleavage are needed. Enzymatically, certain configurations of a peptide provide the correct specificity for cleavage of a peptide bond(s). Several proteases can be employed for this and together with the other techniques outlined can form 'a grand strategy' for the sequencing of most peptides (Fig. 1.1.2 (e)).

Trypsin is a very widely used protease, because it is readily available, highly specific and is active regardless of the length or amino acid sequence of a polypeptide. Chymotrypsin, pepsin and thermolysin are not

Method	Peptide Bond Cleaved
Trypsin	Amino Acid 1 : Lysine or Arginine
Chymotrypsin	Amino Acid 1 : Phenylalanine, Tryptophan or Tyrosine
Pepsin	Amino Acid 1 : Many Incl. Phenylalanine Tryptophan and Tyrosine
Thermolysin	Amino Acid 2 : Leucine, Isoleucine or Valine
Cyanogen Bromide	Amino Acid 1 : Methionine

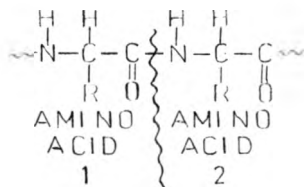


Fig. 1.1.2.(e) Enzymatic Specific Cleavage of Polypeptides and Proteins

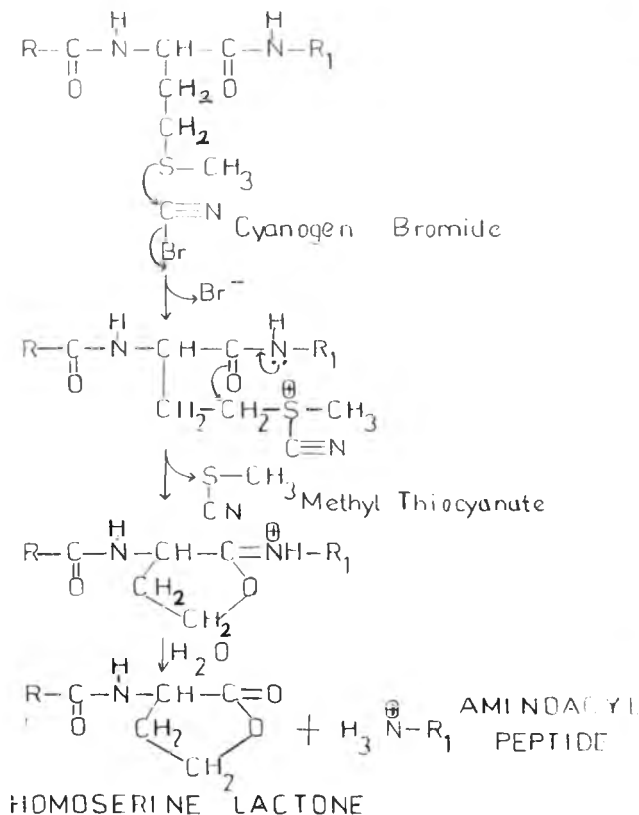


Fig. 1.1.2.(f) Cleavage for a Polypeptide Chain at a Methionine Residue using Cyanogen Bromide

as specific as trypsin but are used in conjunction to provide alternate peptide cleavages for overlap analysis and also where a protein has very few or no arginine and lysine residues and is therefore resistant to trypsin cleavage, or when there are so many (eg. protamines, basic proteins found in cell nuclei) arginine and lysine residues that cleavage by trypsin provides data of little use for sequencing.

Furthermore, specific chemical methods of cleavage of polypeptides have been developed. The most successful, because of its high specificity, involves the reaction of polypeptides with cyanogen bromide. This reagent cleaves peptide bonds whose carbonyl function is contributed by a methionine residue (Fig. 1.1.2. (f)). The methionine residue is converted into a C-terminal homocysteine lactone. As with all of these highly specific cleavage reagents, reaction with a polypeptide yields a known number of fragments if the sequence is known or the number of methionine residues in the protein.

Coupling of 2 or more of these techniques is required to give information required for overlap analysis and subsequent sequencing of polypeptides and proteins. (Ref. 7)

1.1.1 Postulated Method of Sequencing

A possible method of the sequencing of derivatized polypeptides or proteins utilizes a set of enzymes with very specific modes of action but varied specificity, followed by separation of the digestion products by HPLC and identification of the products by mass spectroscopy.

The following sections (1.1.4.1-1.1.4.6) detail the actual instrumentation, chemicals, enzymes and identification theory that could be used in the new method proposed here. Many of the suggestions are based on results obtained in this study or unpublished data obtained during my research.

1.1.4 Advantages of Postulated Method Over Established Methods for Peptidyl-Sequencing

The overriding advantage that this postulated method of sequencing polypeptide chains (see section 1.1.4) lies in its simplicity.

It is hoped that when the method is finally worked-out, that the sequencing of polypeptides can take place routinely in a "one-pot" system with subsequent detection of digestion products from highly-specific enzyme digestion on a versatile HPLC-FAB-MS apparatus. This would (a) save time and hence money, (b) work for all polypeptides irrespective of derivatization or not, (c) require less sample and (d) be highly controllable.

1.1.4.1 Instrumentation

Careful control of the digestion of a given sample of a polypeptide is vital to ensure conservation of materials, time to identify products produced, as and when released, to enable a form of sequencing to be possible, and to give a means of reproducibility. Therefore, a well thermostatted incubator for digestion is required which would be coupled with HPLC systems capable of resolving quite similar digestion products (i.e. dipeptides) enough for their inclusion in separate fractions for separate addition to a mass spectroscopy probe for identification or, utilizing a new probe called a flow-through-probe which allows the eluant from an HPLC analysis to flow straight onto the probe tip to undergo immediate fragmentation and identification.

1.1.4.2 Choice of Polypeptide or protein to be analyzed

The type of protein that this method of sequencing is envisaged as being capable of handling are those that have derivatized amino terminal amino acids, as explained earlier. They must also be available in sufficient quantity for successful digestions and subsequent analysis to be carried-out (down to nano molar quantities required). Furthermore, the tertiary and quaternary structures of the protein or polypeptides must be able to undergo partial denaturation to enable digestion to take place. Lastly, one must be aware of the presence of metal cofactors in the sample which may need to be removed; as one can see in this study, some of the sequencing enzymes used - are inhibited by certain metals.

1.1.4.3 Choice of Enzymes

The choice of enzymes to use must be compatible to the method of identification of digestion products (ie FAB-MS and HPLC) from these enzymes. For example, although Chymotrypsin and Trypsin are well documented enzymes as to their preferred points of digestion, the fragments produced are very large, too large for separation on the HPLC system detailed below and of a size that would make mass spectral analysis a problem. Furthermore, of course, the use of these enzymes does not provide a detailed sequence of the products produced - only the sequence at the ends of the products, but by the use of further site specific enzymes an overlap analysis of the fragment can be made - but this is costly, time consuming and needs more material. The ideal enzymic method of sequencing a protein (especially derivatized) must lie with the inclusion of a group of wide-substrate specific enzymes of the

protease - type called "Cathepsins" or Disiminopeptidases (DAP's). As the latter name implies, these enzymes i.e. DAPI and 'V are capable of cleaving dipeptides from the amino-terminus of an underivatized polypeptide or protein, which can then be isolated by HPLC and analyzed by mass spectroscopy, because a known dipeptide (X-Y) has a known mass (more accurately a known mass to charge ratio of M/Z). Identification of the sequential products would then give a very detailed sequence of the protein studied, right down to the dipeptidyl stage. i.e. Fig. 1.1.4.3(a)

Of course this appears to be quite simple - but two major problems arise. Firstly, how does one take into account the presence of a derivatized (eg. Acetylated) amino acid at the amino terminus and secondly, any dipeptidyl product masses obtained are inherently ambiguous because a given mass would represent the two dipeptides X-Y and Y-X. Therefore, how can one overcome these problems? A second type of enzyme is required that would specifically cleave away the acetylated amino-acid with subsequent identification, thus allowing the dipeptidases to continue the digestion. Acetylaminopeptidase (AcAP) (Chapter 4 and 5 this study) has proved capable of cleaving, not only acetylated amino acids but also formylated amino acids - two of the most common natural derivatizations encountered i.e. Fig. 1.1.4.3.(b).

The second problem can be tackled in at least two ways. Firstly, once the derivatized amino acid has been removed then the dipeptidases can digest the test fragment and give rise to a dipeptide map. Then either chemically or using a fourth enzyme (deacylase) (as in Fig. 1.1.4.3.(c)) the acetyl group can be cleaved leaving an underivatized terminus ready

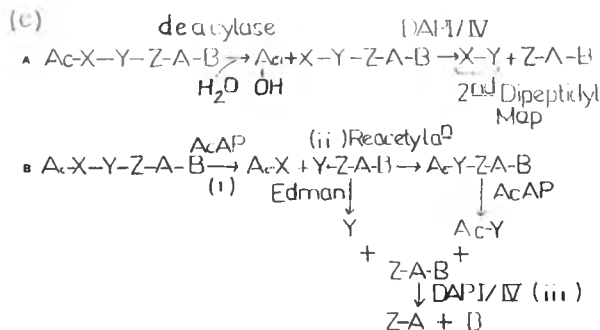
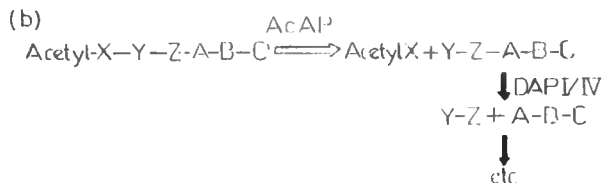
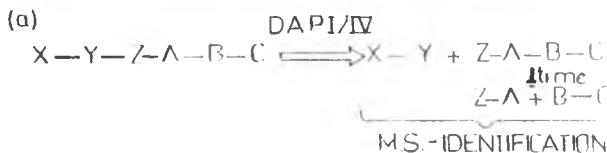


Fig. 1.1.4.3.(a)-(c) Postulated Methods for the Systematic Cleavage of Acetylated or Underivatized Polypeptides

for digestion. Thus, the dipeptidyl map produced would be exactly one amino acid out of step allowing overlap analysis of the maps to be made, giving the accurate sequence. However, if one does not wish to employ a fourth enzyme with all the problems encountered or conditions etc required than this overlap analysis can still be possible by (i) removal of the first acetylated - a.a. by AcAP then (ii) re-acetylation of the fragment at the terminal end followed by a second round of AcAP digestion and (iii) lastly employment of the dipeptidases as before. Thus, once again this method would give a dipeptidyl map - one amino acid out of synchronisation with the original map i.e. Fig. 1.1.4.3.(c).

The above method is theoretically and to some extent experimentally feasible - but has the problems of some of the older methods of sequencing - that of time and material wastage.

Another method of identifying the order of two amino acids in a dipeptidyl fragment is being evaluated. The method consists of the further analysis of the mass spectrum a dipeptide generates by comparison of the intensities of some of the peaks obtained to the hydrophobicities calculated for the dipeptide or by the secondary breakdown of a parent dipeptidyl peak into its daughter ions by a linked-scan facility, by analysing a B/E scan of the products. (see Section 1.1.4.5).

The reason for the choice of both DAPI and DAPIV for the digestion 'cocktail' is attributable to their differing yet complementary specificities (Ref. 7). The specificity of DAPI is limited in that it cannot hydrolyze a peptide bond when a proline residue is involved or if

the polypeptide chain contains an N-terminal arginine or lysine residue. However, DAPIV preferentially hydrolyzes a prolyl containing polypeptide when the proline is in the penultimate position, and has, according to Caprioli and Seifert (Ref. 7), shown a preponderance to release dipeptides from a polypeptide chain with N-terminal lysines or arginines. Thus, by combining the two one has a cocktail theoretically capable of digesting the great majority of polypeptides down to their dipeptidyl components. Only when the remaining polypeptide fragment has a proline residue in position three will cleavage by DAPI and IV stop. Caprioli and Seifert demonstrated this for α -carboxymethylated soybean trypsin inhibitor (Ref. 7). However, by reference to the schemes outlined earlier one can remove the residue in position 1 by deacylation and subsequent cleavage by AcAP (see Fig. 1.1.4.3(c)) or by one cycle of the Edman degradation procedure (see Section 1.1.2), thus allowing the continuation of DAP - digestion and subsequent sequencing.

Finally, the use of DAPI and IV in partnership is only possible because they are compatible in their specificities, but more importantly, because they both exhibit sufficient activity at around pH6.5 to perform hydrolysis and do not interfere with one another's mode of action. (Ref. 7).

Thus, it is for the above reasons that the three enzymes, diamino-peptidases I and IV and acetylaminopeptidase would be employed in this sequencing technique. DAPI and AcAP were purified and characterized in this study. DAPIV was isolated and characterized by Dr A. Woolfitt by a method adapted from Hopsu-Havu et al. (Ref. 8) and published during this research (Ref. 70).

1.1.4.4 Separation of Digestion Products

Post-digestion reaction mixtures have to be separable into their component parts for analysis and quantification of products. The major components, if pure enzymes and samples for digestion are used, would thus be the native sample peptide, digested sample and products and of course the digestion enzyme(s) themselves. The problem then becomes, the ability to separate the digestion product (eg. a dipeptide cleaved from the sample peptide) from everything else. This has two advantages (a) the separation of the digestion enzyme from sample, thus effectively stopping digestion i.e. sequentially cleaving dipeptides at a controllable rate and (b) simplification of identification of the products. (Ref.9-11)

The digestion enzymes used can be relatively easily removed because of their high molecular weight by gel filtration or precipitation. Further, if one uses an immobilization technique (as for DAPI, Chapter 3) then simple filtration can remove the enzyme. The most important separation required, is to remove the native and partly digested peptides. High performance liquid chromatography is a widely used technique for the separation of peptidyl mixtures. In original experiments by Moore & Stein an ion-exchange procedure was used for the separation of free amino acids on sulphonated cation-exchange resin, with quantification and detection by a post-column reaction with ninhydrin. More recently, the development of new column material with very uniform micro-pariculate silica (3-10 μm) chemically modified to give a surface coating with different functional groups. 'Reverse-phase' (RP) packings, in which silica particles are reacted with alkyl-silylating reagents (hydrophobic hydrocarbon chains) and used with polar (principally aqueous) mobile

phases have proved very useful for analysis of mixtures which exhibit both hydrophobic and hydrophilic characteristics.

Few amino-acids have functional groups which enable direct analysis and quantification directly in solution (ie A280 nm). Those that do, ie. phenylalanine, tyrosine and tryptophan owe their UV, fluorescent and electrochemical activities to their substituted, aromatic nuclei. Therefore, if one was trying to analyse and sequence a peptide with direct quantification then many amino-acids/dipeptides would require derivitization by, for example Dansyl chloride. This study is partially aimed at avoiding the need for derivitization by using FAB-MS analyses.

(see section 1.1.4.5).

The high resolution of RP-packing (eg. C18-column) together with gradient elution provide a means to separate most amino-acids or dipeptides using , phosphate buffers with methanol or acetonitrile as organic modifiers. Furthermore, 0.1% trifluoroacetic acid (TFA) is quite often included, which acts as an excellent solubilizing agent.

For this, hypothesized method of the sequencing of polypeptides, HPLC would be utilized using a C18-RP column with elution by 35% Acetonitrile/ methanol +0.1% TFA and normally 65% demineralized water. However, (see section 1.1.4.5), because it is hoped to run eluant from HPLC column chromatography directly into a mass spectrometer then a matrix-forming component in the elution of the column needs to be incorporated prior to chromatography, or added as the eluant flows into the spectrometer.

1.1.4.5 FAB/MS Fragment Identification

It was as long ago as the late 1950's that chemically modified peptides afforded mass spectra from which, primary structures could be ascertained.

The observation that the peptide backbone preferentially fragments at the amide linkage, gave the idea that mass spectroscopy (m.s.) could be used in sequencing studies of proteins. However, at this time such analysis required the need for volatile samples using the conventional ionization techniques. Investigation of free amino acids was therefore difficult and prior chemical modification to enhance volatility was needed.

Permethylation, is a reaction (Ref. 12) which helps to reduce intermolecular hydrogen bonding, and was used in the first full mass-spectrometric primary-structure determination of an unknown protein, dihydrofolate reductase by Morris (Ref. 13).

The use of gas chromatography to achieve prior separation of peptide mixtures was suggested as early as 1960, using O -trimethylsilylated polyamino alcohol derivatives.

However, several ionization techniques have also been developed which allow the production of mass spectra from underivatized peptides. The most successful used in recent years, were field desorption (FD) and plasma desorption (PD) (Ref. 14). FD-MS show abundant parent ion (M) plus H^+ species on the spectra, but does not give fragment ions, and hence, sequencing information in any great intensity. PD-MS utilizes the energetic fission products from the decay of $^{252}\text{californium}$ (Cf) to

volatilize and ionize a solid sample which results in spectra containing peaks attributable to $(M+H)$ and $(M-H)$ species plus extensive fragmentation. Some success has been demonstrated in the applicability of PDMS in high-mass and mixture analysis. (Ref. 17,18)

A third technique is the use of fast-atom bombardment, (FAB) developed by Barber and co-workers (Ref. 19,20) which allows mass spectra to be obtained from involatile and/or thermally labile compounds. Peaks observed include molecular mass peaks and fragment information, in either the positive or negative mode of operation. Sample preparation is routine and can allow sample recovery after the experiment. It involves the dissolution of the material into a suitable solvent matrix. The most commonly used matrices are glycerol and thioglycerol, with added solubility provided by added acids, bases or other co-solvents. Such spectra often show the presence of protonated molecular ions as well as cationized species. However, the presence of too high a concentration of alkali metal ions like sodium and potassium may lead to the suppression of the mass spectrum. As well as the molecular ion peaks; important peaks at the lower end of the spectrum can be present which are characteristic of a particular amino acid. (Table 1.1.4.5).

Naylor et al (Ref. 21) highlights a limitation in the use of FAB mass spectrometry for mixture analysis. That is to say, some components dissolved in matrices like glycerol, are not normally observed. It was shown that it was the most hydrophilic peptides which are suppressed. Furthermore, it was also shown that in analysis of pure hydrophilic and hydrophobic peptide mixtures that, (a) hydrophilic peptides alone give poor signal responses and (b) hydrophilic peptides are further suppressed

Table 1.1.4.5 Some Amino Acid Diagnostic Ions (Ref 16)

Amino Acid	Diagnostic Ion (M/Z)
Tyrosine	136
Histidine	110
Phenylalanine	120
Leucine/Isoleucine	86
Valine	72
Proline	70

in the presence of hydrophobic peptides, that initially occupy the surface of the matrix. Naylor *et al* has shown that it is possible to predict which peptides would be seen in preference to others by the calculation of a peptide's hydrophobicity rating or index obtainable by reference to the scale of Bull and Breese (Ref.22), which relates to the preference or reluctance of an amino acid to transfer from aqueous solution to an air-water interface. The more positive the index the more hydrophilic the peptide. Although the hydrophilic peptide is suppressed in the early stages of FAB - analysis, it is possible to see that as the time elapsed of fast atom bombardment (eg with xenon atoms), the disappearance of the peak due to the most hydrophobic peptide occurs with the appearance of the hydrophilic peptides increasing. This is due to the fact that, as the hydrophobic peptides occupied the matrix surface initially, it is they that are first subjected to bombardment and resultant sputtering away for analysis, thus the suppression of the hydrophilic peptides decreases with time.

To improve on this situation one can do two things. (1) derivatization of the products of an enzymic digestion by converting them to their isopropyl esters tends to equate the hydrophobicities of all peptides, so that every peptide is seen with equal intensity and, at the same time or (2) use HPLC separation of the digests prior to analysis. This latter solution is the one favoured, as it does not involve chemical modification of a sample; and also can be linked in series - directly to the probe of a spectrometer, with constant flow of the eluant from HPLC separation onto the tip of a probe via a hollow probe (ie. Flow-through probe). Work on this technique is very new, and early results give grounds for optimism. This flow through idea, also alleviates a further problem,

in that the intensities of the peaks of a spectrum taken of a sample change with the time on the probe. This fact can be attributed not only to the loss of sample but also to a change in the matrix composition which may favour the release of certain peptides. The flow of the HPLC eluant directly onto the probe provides, chromatographically separated sample components in 'fresh' matrix of an accurately controllable composition.

Therefore as one can see, work in the field of peptide and protein sequencing has been quite extensive recently, and in particular the use of enzymatic digestion of a peptide sample with subsequent analysis by GCMS (Ref.23) or GC-MS (EI) (Ref.24). Not all of the strategy has been fully worked out, but in the very near future it is envisaged that there will be a technique that is both quick and accurate, with minimum sample preparation and which utilises many of the aspects outlined in these preceding sections.

1.1.5.1 Diaminopeptidase I: Pseudonyms, source and History

An intracellular peptidase-type enzyme isolated from bovine spleen, was first described by Gutmann and Fruton (Ref.25) in 1948. In 1952 Tallian et al (Ref.26) gave this enzyme a name, Cathepsin C (E.C. 3.4.4.9). Several papers showed that this enzyme had very limited substrate specificity (Ref. 27,28).Metrione et al (Ref.29) used the term Dipeptidyl transferase, a name adopted by Voynick and Fruton (Ref. 30) in 1968, which highlighted this enzyme's function at alkaline pH. McDonald et al (Ref.31) published the first of a series of papers in 1965 which investigated the properties of a chloride-activated sulphhydryl-dependant enzyme from bovine pituitary gland similar in action to the bovine spleen Cathepsin C. This enzyme was later found to be capable of releasing dipeptides (ie. initially from the substrate Serine-tyrosyl-naphthylamidase) sequentially from polypeptides and was termed dipeptidyl aminopeptidase I (DAPI, E.C.3.4.14.1). Subsequent work with the spleen Cathepsin C lead to the conclusion that it was identical with the pituitary enzyme (Ref.32). This name highlights the enzyme's action at acidic pH, and the latter name, DAPI is more commonly adopted (as in this study) and the fact that it has a transferase activity at alkaline pH is not overlooked, but is of smaller importance than the dipeptidase activity shown.

Liver has already been reported as the richest source of DAPI in rat (Ref.33) but it has also been found in many other tissues, and this fact has been attributed to its physiological function. It is highly likely that DAPI is located in the lysosomes of tissues. However, although Shibko and Tappel (Ref.34) could not detect DAPI in kidney lysosomes,

and further work could not detect DAPI in any other sub-cellular fractions it seems likely that the method of detection was very insensitive, since separate findings confirm that rat kidney is approximately 25% as rich in DAPI as liver.

Similar findings have been observed in other animal tissues, in particular bovine spleen and pituitary glands.

1.1.5.2 DAPI: Physical Properties

DAPI is a chloride activated sulphydryl - dependant enzyme, which, it is postulated, forms dipeptidyl - enzyme intermediates when exhibiting its dipeptidyl cleaving action.

The pH-dependence of DAPI depends upon the substrate (see section 1.1.5.3) to be used, but, in general, it has a pH-optima range of 5-6 for its dipeptidyl action, but also a pH - optimum of pH 6.8 for its transferase action. Since all enzyme catalysed reactions proceed to an equilibrium position it has been postulated that DAPI may well exhibit a reverse - dipeptidyl activity at more alkaline pHs. This idea has been investigated to some extent by the monitoring of the polymerization of dipeptide amides typically Gly-Phe NH₂ and Gly-Trp-NH₂, and for some basic substrates, such as Gly-ArgNH₂ and Gly-Arg- β -naphthylamide up to the size of (GlyArg)₅ β -naphthylamide (Ref.31). More acidic pH-values (Below pH 3.5) are unsuitable because the enzyme is rapidly and irreversibly inactivated.

Metrione and Okuda's work on DAPI, indicates that there are 8 sulphhydryl groups per unit of 210,000 the particle weight of the pure protein reported by Metrione et al (Ref.29). McDonald reports similar molecular weights for DAPI, namely 200,000 by Sephadex chromatography for the rat liver DAPI and $210,000 \pm 30Kd$ reported by Planta and Gruber for the bovine spleen DAPI determined by sedimentation equilibrium experiments.

The isoelectric point of DAPI was determined by gel electrophoresis under conditions that preserved the enzyme's activity (cf section 4.3.3.3, AcAP) Activity Stain, this study) and a value of pHS was reported by McDonald et al (Ref.31).

McDonald et al (Ref.31) have shown that when the rat liver Cathepsin C was treated with 2-Mercaptoethanol (1%) and sodium dodecyl sulphate (1%), according to the method of Shapiro et al (Ref.36) this resulted in the apparent dissociation of the enzyme. After analysis of a dissociated sample by electrophoresis, two bands were detected, the slower component was estimated at a weight three times larger than the faster component. The results of sedimentation studies by Metrione et al (Ref.29) also suggested a possible polymeric structure for the bovine spleen Cathepsin C (DAPI). They also found two subunits with a mass ratio of 3.

1.1.5.3 Substrate Specificity

The broad specificity of DAPI was first demonstrated when β -corticotrophin was used as a substrate. At pH 5.5 preparations of DAPI, whether purified from beef spleen or pituitary glands or rat liver; catalyzed the successive removal of dipeptides from the amino-

terminus of the hormone: DAPI can remove dipeptides having penultimate residues with both hydrophilic and hydrophobic side chains. The most susceptible dipeptides were those which contain penultimate basic residues eg. Arginine and lysine. However, as demonstrated by Izumiya and Fruton dipeptidyl amides where the first or both residues are arginine or lysine were shown to be immune to attack by DAPI. On the other hand, when a substrate contains a group of residues with acidic side chains; then this also seems to retard DAPI's action as demonstrable by the action of DAPI on Glutamate tetramers. However, this problem can be overcome to some extent, by lowering the pH of the reaction to about 4.0-4.5, to suppress the ionization of glutamyl or aspartyl residues. Proline is another residue, which when present can cause the cessation of DAPI's action. If the proline residue is the second or third residue in a peptide, than cleavage does not occur ie. DAPI appears not to hydrolyze the amide bonds on either side of proline. If, however proline is the first residue than hydrolysis does occur with the rates of hydrolysis of the two substrates Pro-Phe-NH_2 and Gly-Phe-NH_2 being highly comparable.

The size limits of the substrate ie. number of residues in the chain, appears to have no upper limits, provided the amino-terminus is accessible to DAPI. Action on several peptides has been demonstrated with the complete hydrolysis of the A-chain of reduced (Carboxy-methylated) bovine insulin, a 21-residue peptide and the release of 13-dipeptides from the amino-terminus of the B-chain of oxidized bovine insulin being two good examples. Other substrates, eg. glucagon, **β** -corticotrophin, angiotensin II (Human) highlight the facts that the presence of double - arginine residues or proline in positions 2 or 3,

halt DAPI hydrolysis (see earlier). In general, there appears to be no lower limits for the size of a peptide to be hydrolyzed but it is worth noting that some tripeptides eg. (Phe)₃ and (Gly)₃ are hydrolyzed at slow rates, and (Ala)₃ is resistant. On the other hand (Phe)₄, (Gly)₄ and (Ala)₄ are hydrolyzed readily into dipeptides. (Ref. 31)

There are several reports which utilize different substrates with β -naphthylamide, p-nitroanilide, amide, and ester derivatives; most have been used as a means of detecting and quantifying DAPI, with varying sensitivity - but the underlying trend follows the trends noted earlier, and indeed any dipeptide (with mentioned exceptions) that has been derivatized by a colorimetric, fluorogenic or other detectable property group, after hydrolysis, could be used as a possible substrate for DAPI under the correct conditions.

1.1.5.4 DAPI: Storage and Stability

McDonald et al (Ref.31) quote Metrione et al (Ref.29) when they studied the storage of DAPI. The latter workers state that DAPI did not withstand freezing or lyophilization and consequently both groups of workers stored their preparations of DAPI on ice. The rat liver preparation of McDonald et al was found to be relatively stable indicated by a loss of activity of only 5% in the first month of storage in 1% sodium chloride and 4mM 2-mercaptoethanol. However, McDonald et al in their publication (Ref.31) appear to have found a method for the stable storage of DAPI as a lyophilized powder. Their preparation from bovine spleen was treated with 1% EDTA as well as 1% sodium chloride and 4.0mM 2-mercaptoethanol, because this acted as a dipeptidase inhibitor,

which if the preparation were to be used in sequencing studies, would, in time, cause partial breakdown of liberated dipeptides. Another contaminant enzyme in their preparations was a carboxypeptidase which attacked the carboxyl residues adjacent to a proline residue. Diisopropyl phosphorfluoridate (DPP) was included in the purification procedure, just prior to ultrafiltration and lyophilization, which effectively acted as a carboxypeptidase inhibitor. They then freeze-dried their preparation in 0.2ml aliquots (4mg Protein) and sealed them under vacuum. Up to 10% of the activity may be lost in this way, but the remaining activity was found to be stable under refrigeration for nearly two years. Each ampoule upon reconstitution in 2 mls of 1% sodium chloride - 1mMEDTA and stored on ice was also found to be relatively stable, losing about a further 10% activity in a month.

The stability of DAPI preparations were variable when subjected to various purification methods. Planta and Gruber (Ref.35) reported that several methods (including ammonium sulphate precipitation) of ultrafiltration or concentration of samples were found to result in huge losses of enzyme activity. It is for this reason that when concentration is required then ultrafiltration on an Amicon cell using UM-10 filters is the most commonly used method in the preparation of DAPI, since this appears to reduce to a minimum the loss of DAPI when concentrated.

Apart from the obvious requirements (see section 1.1.5.5) that DAPI requires for full activity, temperature and pH are important factors when the stability of a preparation is considered. As can be seen, many purification methods (Ref.35, and this study) of DAPI utilize a heat

step. DAPI appears to be stable to 65°C for anywhere up to 40 minutes, with optimum activity occurring at about 37°C and slight losses of activity occurring at about 0°C.

The pH at which DAPI is assayed, between 3.7-8.5, is possible because the enzyme is stable over a wide range, partly shown by the different types of activity it exhibits. However, below pH 3.5 and above pH 8.5, preparations of DAPI lose activity rapidly and irreversibly.

1.1.5.5 DAPI: Inhibition and Activation

As has been outlined earlier, the halide requirement of Cathepsin C (DAPI, Ref. 38) appears to be absolute. Sulphydryl activation was only possible in the presence of a halide. Chloride ions gave the maximum rate when used in Gly-Phe- β -naphthylamide hydrolysis, at concentrations of between 5 and 10mM, and many substrates tested showed a pre-requisite for chloride ions before action. Other halides tested, i.e. bromide and iodide ions were also relatively effective as activators, whereas fluoride ions were of little effect. Furthermore, nitrate ions in a similar way with the angiotensin "converting enzyme" (Ref. 31) also satisfied the halide requirement; as do thiocyanate, and chlorate ions (Ref. 38).

Gly-Phe-amide has been used to inhibit the hydrolysis of Gly-Phe- β -naphthylamide but far higher concentrations of the "inhibitor" were required (445x) to achieve only 33% inhibition (Ref. 29). This result indicates that Gly-Phe-amide was acting as an alternative substrate and thus caused "inhibition" and it further indicates the preference that DAPI has for the naphthylamide substrate over the amide at that pH.

At saturating activator concentrations, substrates are split according to Michaelis-Menten kinetics. At non-saturating concentrations co-operativity is observed. Accordingly, in the absence of activators, a low concentration of the substrate Ala-Phe-amide enhances the rate of hydrolysis of Gly-Phe-p-nitroanilide. It is of interest that, for DAPI high substrate and activator concentrations are inhibitory.

Copper (II) chloride (Cu^{2+}) inhibition is observed at pH5 using a 0.1mM solution at 37°C after a ten-minute incubation. This led to a decrease in the activity of the enzyme to about 50% of the value of the control tube. (Ref.30).

Diazoacetyl amino acid esters are a class of compounds which resemble, structurally, the substrates of DAPI and were postulated as possible inhibitors. With pepsin, these compounds do effect some inhibition, but only in the presence of an added metallic catalyst, like the cupric ion. No added inhibition of DAPI was seen, even in the presence of cupric ions, and this finding may give some evidence as to the mechanism of action of DAPI, in that it may have a different mode of action to pepsin. It was also noted that although inhibition did not occur using the diacetyl amino acid esters, hydrolysis did not occur, which highlights the specificity of DAPI for unblocked amino-terminal amino acids as substrates.

1.1.6.1 AcAP: Pseudonyms, Sources and History

There are many pseudonyms and former names for AcAP arising from the fact that this enzyme has been isolated from many sources - each time been given a different name - relating to type of reaction exhibited. Only recently has each of these properties been collated and attributed to the same enzyme, AcAP (E.C.3.4.14.3).

Tsunasawa et al, reported the purification of an Acylamino acid-releasing enzyme in 1975 (Ref. 41) closely followed by analysis of the enzyme from rat liver in 1976 (Ref.42). They tied in the connection their enzyme had with a similar enzyme found by Yoshida and Lin in 1972 (Ref.50) in rabbit reticulocytes.

In 1978, Gade and Brown (Ref. 45) isolated an α -N-Acylpeptide Hydrolase enzyme from bovine liver, the properties and physical characteristics of which appeared to resemble the rat-liver enzyme of Tsunawasa. A property of AcAP was highlighted by Suda et al (Ref.44) in 1980, when they purified an enzyme from rat liver that exhibited a high specificity for the release of a formyl-methionine residue from f-met-peptides, hence the name given f-met-Aminopeptidase. It has since been realized that this enzyme is the same as the Acylamino Acid - releasing enzyme of Tsunawasa, but exhibiting a different property under different conditions. Other names given to AcAP include formyl-methionine- releasing - enzyme, N-acetylalanine - aminopeptidase and N-acetylaminosacyl-p-nitranildase. Furthermore, the classification of this enzyme has recently been altered to that mentioned above (Ref. 40).

There are many sources of AcAP. The enzyme appears to be cytosolic, showing high levels in liver, spleen and reticulocytes; moderate levels in erythrocytes and lung; and low levels in muscle, heart and brain of the rat (Ref. 41,42) It has also been shown to be plentiful in the cytosol of human erythrocytes, but is absent from leukocytes (Ref.43). Other sources include beef (Ref.45) and hog livers (Ref.46), human placenta (Ref.47); and sheep erythrocytes (Ref. 37).

1.1.6.2 AcAP: Physical Properties

The molecular weight of AcAP has been reported at anything between 290,000 by Suda et al obtained on gel filtration using Sepharose CL-6 B to 423,000 by Taunasaawa and Narita (Ref. 42) obtained by sucrose density gradient ultracentrifugation, although it is worth noting that the latter paper also contains a second method of molecular weight estimation, by gel filtration and quotes a molecular weight of 360,000. Gade and Brown give a figure of 320,000 for its molecular weight (Ref.45) and that it is a tetramer. Taunasaawa et al (Ref. 41) describe AcAP as a pentamer or hexamer with identical subunits of approximately 75,000 each. This latter value is based on the high molecular weight estimation quoted in their paper of 423,000 obtained by sucrose density studies. Suda et al also describe AcAP as a tetramer with each subunit having an equal weight of about 72,000 determined by SDS-electrophoresis. Whatever the exact number of subunits present it seems certain that all of the subunits are identical.

Other molecular weights quoted include 300,000 for the human red blood cells by Schönberger and Tachasche (Ref. 43) and 380,000 for the human

placental enzyme (Ref.47). This latter enzyme is interesting because it appears to exist in two forms. This is shown by the fact that it has two isoelectric points (IEP) of 3.9 and 4.5. A figure of 4.25 for the isoelectric point of the rat liver enzyme (Ref.41) by Tsunasawa et al has been quoted. Of course, this figure could represent the close migration of two bands, as for the placental enzyme mentioned above, but the method of IE Point determination used by Tsunasawa et al may have failed to distinguish the two.

1.1.6.3 AcAP: Substrate Specificity

It seems highly probable that AcAP is involved in the catabolism of many soluble proteins in eukaryotic cells, since a figure of 80-90% of all of these proteins appear to be N-acetylated (Ref.49). However, Jones and Manning (Ref.52), in 1985 using the name of Acylpeptide hydrolase for AcAP hypothesized that the human erythrocyte enzyme was possibly important in polypeptide processing during biosynthesis. Brown and Robert (Ref.49, 1976) thought that the presence of acetylation on proteins and polypeptides marked them for breakdown. This may have some credibility since Shiffman (Ref.71) has shown that L-methionine peptides (another substrate of AcAP) could act as chemoattractants for leukocytes. In general, AcAP appears to be capable of cleaving a wide range of acylamino acids from N-acetylated peptides, but does not show any action on N-Acetylated amino acids (Ref. 45) once cleaved, Pyroglutamyl peptides (Ref.44), or N-Ac-hexosamines (Ref.45). Although some examples have been found, in general non-acetylated peptides are not attacked. Acetylated peptides have been shown to be hydrolyzed 4x, 8x and 15x faster than the corresponding formyl, propionyl and butyryl derivatives respectively (Ref. 45).

Studies on the effect that the number of residues in an acetylated peptide has upon the rate of hydrolysis by AcAP has shown that there is a significant change with the lengths. A number of Acetylated polyalanine peptides i.e. Ac(Ala)₁ to Ac(Ala)₆ were tested by subjecting them to hydrolysis with AcAP. Gade and Brown found that AcAP preferred Ac(Ala)₃ (100%) as a substrate with Ac(Ala)₂ as the most unfavourable (7%). (Note: Jones and Manning (Ref.52) quote a figure of 50% for the hydrolysis of Ac-(Ala)₂ as opposed to 7%, but still show that the acetylated-dialanyl residue to be the worst substrate). Ac(Ala)₄ and Ac(Ala)₅ show rates of hydrolysis to be 58% and 62% of Ac(Ala)₃ hydrolysis rate, respectively. Other trialanine peptides which had been derivatized by formylation, propionylation and butyration show relative rates compared to Acetyl-(Ala)₃ to be 26%, 12% and 8% respectively (Ref.45). N-Ac-Met-Thr (N-Acetyl-L-methionyl-L-threonine) (Km 0.8mM) has been a widely used substrate of AcAP, the release of threonine being quantitatively detected by the ninhydrin colorimetric assay of amino acids developed by Yemm and Cocking (Ref.51). Direct colorimetric assays of AcAP have been developed including p-nitroanilide derivatives of AcAla and β -naphthylamide derivatives of L-methionine. The comparisons of the rates of acetylated peptidyl hydrolysis by AcAP when compared to Ac-Met-Thr is shown below; Ac-Met-Ala-95%, Ac-Ala-Tyr-79%, Ac-Ser-Tyr-67%, Ac-Ser-Thr-NH₂-65%, Ac-Leu-Ala-Gly-62%, AcMet(Me)-38%, Ac-Met-Thr-Ome-29% and Ac-Met-NH₂-5%. No action was seen when the D-forms of amino acids were involved. (Ref.41). These values were obtained on substrate concentrations of 2mM at 37°C and pH 7.2.

The intended use of AcAP in this study as a "starter-enzyme" in the

hydrolysis of acetylated or formylated peptide samples gains credence by the fact that this enzyme has already been involved with some work in this area by Tsunasawa et al (Ref.41) and Nakamura et al (Ref.48) when they used it to initiate the sequence analysis of an octapeptide containing N-Acetyliserine.

1.1.6.4 AcAP: Stability and Storage

AcAP has shown some reactivity and stability between pH 5 and 9.2. (Ref. 43). The pH-optimum range of AcAP for many substrates is pH8-8.3. Gade and Brown have claimed that AcAP can exhibit activity down to pH 4.5, but the majority of workers indicate a rapid loss of activity below pH 5.

The temperature at which AcAP is stored is important. Tsunasawa and Narita (Ref. 42) show that AcAP appears quite stable at high temperatures, since a heat-treatment step is utilized in its purification with the enzyme preparation being submitted to a temperature of 60°C for 5 minutes. Alternatively, if stored at -20°C, -80°C and room temperature, samples of AcAP lose 91%, 16% and 22% of their activities after 2 weeks (Ref.45). In the presence of 2-mercaptoethanol AcAP loses its activity completely when frozen or lyophilized. In its absence, AcAP loses 50% of its activity upon lyophilization. Furthermore, Gade and Brown (Ref.45) have shown that AcAP exhibits a retention of activity of up to 50% when in the presence of high concentrations of the normally denaturing promotor, Urea (4M). Storage of the enzyme solution containing 10mM - 2 mercaptoethanol and 1mM EDTA at 0-4°C for one month results in only a 10% loss in activity (Ref.42).

1.1.6.5 AcAP: Inhibition and Activation

When studies with naturally-occurring peptides are carried-out it seems that chloride-ions have been found to be an effective modulator of AcAP's activity. The activity of AcAP increases when the concentration of sodium chloride is raised. After a concentration of 0.1M, then activity drops off, as more sodium chloride is added. (Ref.53). This observation was observed when the substrates, chloroacetyl-Gly-Leu, Acetyl-(Ala)₃ and Acetylalanyl-pnitroanilide are used. However, Suda et al report that the activity of AcAP increases when concentrations of up to 0.4M sodium chloride are used. The difference may be attributable to the fact that the latter workers performed their studies using a β -naphthylamide substrate at pH 7-8 using the rat liver enzyme. Whereas, Jones and Manning used Ac-Ala-pNitroanilide as a substrate at pH 8.3 and the human erythrocyte-derived enzyme.

Sulphydryl compounds (ie. 2-mercaptoethanol or dithiothreitol) show slight activation effects, and some common sulphydryl - blocking reagents such as mercuric chloride (HgCl_2), p-chloromercuribenzoate (1mM) showed complete inhibition. Other sulphydryl blocking reagents such as iodoacetic acid and moniodoacetamide have little or no effect. This effect may be explained by the fact that Hg^{2+} ions inactivate AcAP completely, and the inhibitory effect of HgCl_2 may not be due to its sulphydryl-blocking role, but due to straightforward inactivation by a divalent heavy metal ion. (Ref.73). Other chemical inhibitors found include 2,2'-dipyridyl disulphide, which together with p-chloromercuribenzoate probably don't act in its sulphydryl-blocking reagent mode but rather as denaturing agents. (Ref.43). Diisopropylfluorophosphate (Ref.73) has

some effect, as does p-chloromercuribenzoic acid (Ref.45) but soybean trypsin inhibitor (Kunitz) has no effect. (Ref.44). EDTA has no effect, and divalent cations are not required for activation. (Ref.41,44). The effects of protease inhibitors of microbial origin were also investigated by Suda et al. Leupeptin, antipain, pepstatin, chymostatin, phosphoramidon, elastatinol, bestatin and amastatin at concentrations of 0.1mM did not show any inhibition.

AcAP exhibits a loss of activity when inhibited by metal ions. Mont workers on AcAP state that AcAP is inhibited completely by Hg^{2+} and Cu^{2+} ions (see also section 4.4.3.5, this study). However, Suda et al. differentiates between the two ions by stating that Cu^{2+} is only a moderate inhibitor, and not as potent as Hg^{2+} , and also that Cd^{2+} (Cadmium) is another potent inhibitor similar to Hg^{2+} . Other moderate inhibitors include Fe^{2+} and Zn^{2+} (see section 4.4.3.5) and no inhibition was observed with Mg^{2+} , Ca^{2+} , Sr^{2+} , Mn^{2+} , Co^{2+} , and Ni^{2+} . All studies used ion concentrations of 0.1mM in the assay of AcAP (Ref.44).

Thus as one can see AcAP is affected by a wide range of inhibitors and activators, and this fact gives a means whereby accurate identification of AcAP in a preparation can be obtained. It is this idea that dictated the methods of characterization of AcAP purified in this study (Chapter 4) along with IE Point and molecular weight determinations and also some substrate specificity studies (Chapter 5).

Section 1.2 Thesis Notation

This thesis is set-out in an alphanumerical form. The sub-sections of the chapters are numbered sequentially up to four sub-sections, after which, each section is given the title (a), (b), etc. Tables or figures are numbered with the same number as that of the section to which it pertains.

References, unless written in full, when referred to are numbered sequentially. No attempt is made to order the references alphabetically or chronologically.

CHAPTER 2

Purification and Characterization of DAPI

Section 2.1 Introduction

Diaminopeptidase I (DAPI) has been studied quite extensively. The aim of this chapter is to purify this enzyme from bovine spleen for use in substrate hydrolysis studies. Of course, once apparently purified this enzyme preparation needs to be accurately characterized for use in the sequencing studies. Much of this chapter details the methods used to characterize this enzyme and to positively identify DAPI. Chapter 3 then makes use of DAPI in an immobilized form to digest a biologically active peptide.

The method of purification has been adapted to include the use of TSK-HPLC. Although an analytical set-up was used - this step could easily be scaled-up to preparative amounts.

Two figures in close agreement for the molecular weight of my enzyme sample are in turn in close agreement to those quoted in literature for DAPI.

The IEF point estimation although not obtained using the latest technology gives a value close to the literature values and as such adds further evidence to the case for my enzyme sample being DAPI. Lastly, the assays used and the storage properties of my sample match those normally attributable to preparations of DAPI. Thus this method can be

said to be capable of producing a sample of DAPI suitable for use in substrate/peptide hydrolysis studies.

2.2 Materials

2.2.1 Purification of DAPI

Fresh bovine spleen (2-3hrs post-kill) was obtained from a local abattoir. Final purification of this enzyme was carried-out using a TSK-G-3000SW Column plus Guard column (300mmx7.5mm) obtained from Amicon Ltd, Stonehouse, Glos., UK, on a Waters Associates model 6000A pump, a U6K injector and a 441UV detector set at 280nm.

2.2.2 DAPI Assays

Glycyl-phenylalanyl amide and Glycyl-arginyl-pnitroanilide were obtained from Sigma Chemical Co. Ltd., Dorset, UK. Glycyl-phenylalanyl-7-aminomethylcoumarin was obtained from Universal Biologicals. Absorbances were measured on a Pye Unicam SP1800 double-beam spectrophotometer and fluorescence measured using a Perkin Elmer L11-3 fluorescence spectrophotometer.

2.2.3 Characterization of DAPI

2.2.3.1 Molecular Weight Estimation

(a) TSK-HPLC estimation

The TSK system used was identical to that mentioned in Section 2.2.1.

Molecular weight marker compounds were purchased from BDH chemicals, Poole, Dorset, UK.

(b) PAGE-Electrophoresis Estimation

Molecular weight marker compounds were purchased from BDH chemicals, Poole, Dorset, UK.

2.2.3.2 Isoelectric Point Determination

Ampholines from LKB (pI3-10) Cathepsin C (DAPI) in 50% glycerol from Boehringer Mannheim.

2.3 Experimental

Based upon a method by Metrione et al and cited in Sequence Determination, Chapter 22, p272 by McDonald et al. (Ref 37).

2.3.1 Purification of DAPI

2.3.1.1 Extraction and Homogenization

Bovine spleen (1.5kg) was skinned and cut into thin strips which were passed through a Waring blender twice. The homogenate (1kg) was dispersed into doubly-distilled water (ddH_2O , 2l) containing disodium EDTA (1.5mM).

The magnetically stirred solution was adjusted to pH3.5 by the addition of sulphuric acid(6N, 22mls), and then readjusted after 1hr (52mls). The solution was then incubated at 37°C for 22hrs (the solution being continuously stirred for the first 5hrs). A clear acid-extract of spleen was obtained by centrifugation of the acid-hydrolyzed solution on a MSE-18 centrifuge at 5°C. This was achieved by spinning at a low speed (3000 rpm) for 20mins and then a high spin (10,000g¹ for 30mins on a 6" rotor.

2.3.1.2 Ammonium Sulphate Fractionation and Dialysis

The apun acid extract (2.18l) was brought to 40% saturation by the addition of ammonium sulphate (243g/l) and allowed to stand for 2hrs. The precipitate was removed by centrifugation at 10,000g for 30min. The supernatant was then brought to 70% saturation by the further

addition of ammonium sulphate (total 440g). After complete mixing the solution was allowed to stand overnight (≈ 16 hrs). The required 40-70% cut protein was collected by centrifugation. at 10,000g for 30min and recovered into a final volume of 900mls of ddH₂O.

The concentrated sample was then dialyzed extensively against a sodium chloride solution (1% w/v) with three 5l changes of the same solution.

The dialysis tubing (10kdalton cut-off) was prepared by boiling in a sodium bicarbonate (1%) and EDTA (trace, Na₂ salt) solution and then rinsed in a boiling ddH₂O bath. The resultant dialysate was clarified at 20,000g for 15mins after adjustment to pH5. (Total Volume = 150mls).

2.3.1.3 Heat Treatment and Ultrafiltration

The dialyzed enzyme solution was dispensed into 15x10ml aliquots in glass test-tubes (suitable for centrifugation) and heated to 60°C in a water bath for 40min, and then quickly chilled on ice. The precipitated protein was removed by centrifugation (10,000g for 30min) and the supernatants pooled.

The heat-treated solution was concentrated (10x) by the use of a Diaflo. Ultrafiltration device from Amicon Corporation using a UM-10filter with a molecular weight cut-off of 10,000 daltons (65ml volume). Further dialysis was carried-out against NaCl (0.1M) plus 2-mercaptoethanol (2mM) and sodium acetate (0.1M) buffer (pH4.5) to equilibrate the enzyme solution prior to the gel-filtration step.

2.3.1.4 Gel Filtration Chromatography

The enzyme solution was applied as a discrete band (15mls) to a column of gel filtration media, Sephacryl S-300 Superfine (495x40mm) which had been equilibrated with the above buffer. Eluant fractions were collected every 125 drops (46mls) into a total of 190 tubes. The eluant was monitored at 280nm. The tubes were tested for enzymic activity (see below) by the Glycyl-phenylalanyl-7AMC fluorescence assay (section 2.3.2.2). Activity was detected in tubes 40-70 and the contents of these tubes were pooled for further purification (180mls), and ultrafiltered down to 20mls as in section 2.3.1.3 above

2.3.1.5 TSK-HPLC Purification

The partially purified DAPI is fully purified by TSK-HPLC gel filtration. Aliquots (10ul of a 29mg Protein/ml DAPI) was injected onto a TSK-gel HPLC column on a Waters system (see earlier). Elution was carried out under the following conditions; flow rate - 0.8mls/min, detection - 280nm, flow pressure - 200psi. Elution was achieved using potassium phosphate buffer (50mM, pH7) containing 2-mercaptoethanol (2mM). The elution profile was obtained and samples (400 µl) collected across it were assayed fluorometrically as described later (section 2.3.2.2). The peak attributable to DAPI was identified and enzyme containing fractions combined. On subsequent runs, these fractions of identical profiles were also combined. Re-chromatography under identical conditions was carried-out to determine the pooled enzymes fractions purity. Combined samples were again ultrafiltered on Centricon 10k dalton cut off devices to 1/10th the volume.

2.3.2 DAPI Assays

2.3.2.1 DAPI Transferase Assay

This assay is based largely upon that originally described by Mentrone et al (1966, Ref: 29), and has since been used by many workers including McDonald et al (Ref: 37). The description of the enzymic unit described by McDonald et al (Ref: 37) was that one unit formed 1 μ mole of hydroxamate/min under the defined conditions assumes similar spectrophotometric properties of the formed dipeptidyl hydroxamate to the standard used, benzoyl-L-alanyl-hydroxamate. In practice, the assay utilizes the fact that the extinction coefficient of the iron salt of hydroxamate is very similar to that of the formed dipeptidyl-hydroxamate and is therefore used as a standard.

The assay is based upon the formation of dipeptidyl-hydroxamic acids (i.e. Glycyl-phenylalanyl -hydroxamate) and the spectrophotometric monitoring of their formation at 510nm. However, care has to be taken that absorbances do not exceed 0.25 after 10 minutes of incubation so as to avoid rate-limiting substrate concentration factors i.e. to keep the assay linear with time.

The following reaction procedure was followed for DAPI assay:-

- (a) For each sample to be tested, the following assay mixtures were set up - to hydroxylamine hydrochloride (NH_2OH , 2M, 100 μ l, pH6.8) was added 2-mercaptoethanol hydrochloride (0.125M, 100 μ l), glycyl-phenylalanyl-amide (0.25M, 100 μ l, pH6.8), ddH_2O (100 μ l) and finally to initiate the assay, the enzyme sample (100 μ l). Total volume = 500 μ l.

- (b) The assay mixtures were incubated at 37°C for up to 15mins.
- (c) Aliquots (100µl) of the incubated mixtures were taken after 0,5, 10 and 15 min. A blank (no enzyme added) was also taken for 'background' reading.
- (d) To stop the reaction, each aliquot was added to 20% trichloroacetic acid (TCA, 500µl).
- (e) 5% Iron (III) chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) in hydrochloric acid (0.1M, 500µl) was also added to each sample and the resultant purple-blue colour allowed to develop.
- (f) The total volume of samples were adjusted to 2.0mls with ddH_2O , and their absorbances read at 510nm.

To test the validity of this assay, known amounts of commercially available DAPI (Boehringer) in 50% glycerol was assayed by the above method and the results obtained are presented in section 2.4.2.1.

2.3.2.2 Fluorescence Assay

The procedure outlined in this section was used to determine the maximal velocity (V_{max}) and K_m - value of glycyl-phenylalanyl-TAMC as a substrate, under the conditions given, and to test the assay effectiveness for DAPI determination.

This assay utilizes the dipeptidylamidase activity of DAPI to cleave the peptidyl bond between the fluorescent label and glycyl-phenylalanine. The following protocol was employed for each enzyme sample to be assayed:-

- (a) To cacodylic acid buffer (0.9M, 10mM) adjusted to pH6 with sodium hydroxide (0.1N) was added,
- (b) 2mercaptoethanol HCL (8.75×10^{-7} M, 12 μ l. Final concentration in assay, 10nM), to satisfy the sulphhydryl and halide requirements for DAPI.
- (c) Glycyl-phenylalanyl-7AMC (13mM in cacodylate buffer, 0-32 μ l).
- (d) ddH₂O to a final volume of 0.99mls and
- (e) finally the enzyme sample was added, a DAPI solution (12 mg Protein/ml, 10 μ l samples added) prepared as in section 2.3.1 of this chapter.

The assay tubes were vortex-mixed, covered with parafilm and incubated at 37°C for 60min.

Blank tubes were also set-up, they were:-

- (i) no enzyme added. To give a measure of background fluorescence and spontaneous hydrolysis of the substrate under these conditions, and,
- (ii) no substrate added, but did include enzyme samples to determine enzyme and assay component fluorescence.

The fluorescence of an aliquot (200 μ l) from each sample was measured with Ex = 380 nm and Em = 442 nm. These excitation and emission values were chosen because they correspond to spectral peaks in 7-AMC's absorbance and fluorescence spectra respectively.

pH-Dependence of the Fluorescence Assay

- (a) Cacodylic acid (10mM, pH3.74) was made up. Sodium hydroxide (0.1N) was added to separate aliquots to give a range of cacodylate buffers between pH3.74 and 8.6.
- (b) Each buffer was then substituted for the pH6 buffer used in the above assay.
- (c) A final substrate concentration of 104 μ M was used throughout, and the assay tubes were incubated at 37°C for 1hr before measurement.
- (d) A control tube for each pH-buffer tested was also set-up and was designed to give measurements of the spontaneous production of 7AMC and/or enhancement of fluorescence at each pH. No enzyme samples were added. Fluorescence measurements were taken as before.

2.3.2.3 p-Nitroanilide Assay

This assay utilizes the dipeptidyl activity of DAPI to hydrolyze the peptide bond between the glycyl-arginyl and p-nitroaniline groups of the substrate, gly-argpNA (see Fig. 2.4.2.3)

The following protocol was used to assay each enzyme sample;

- (a) To cacodylate buffer (10mM, pH6, 580 μ l-containing sodium chloride (1% w/v), EDTA (1mM) and 2-mercaptoethanol (2mM) was added:-
- (b) Glycyl-arginyl-pnitroanilide (10 μ l, 5mM).
- (c) The assay mix and samples were incubated at 37°C (In a thermostatted spectrophotometer) for 10min to temperature equilibrate.
- (d) The enzyme sample (10 μ l) was added and mixing performed by inversion.

- (e) The absorbance at 385nm was monitored on a linear chart recorder.
- (f) The rate ($\Delta A_{385nm}/\text{min}$) was calculated and used to calculate the specific activity of the enzyme sample in conjunction with protein estimations of the samples.
- (g) The number of units of enzyme activity could be calculated in two ways. A calculation derived from first principles using a standard p-nitroaniline curve and secondly a direct comparison between a sample's rate and that of a known and quantified enzyme sample. A comparison of the two methods appears in the results section 2.4.2.3.

K_m and V_{max} Value Determination for DAPI using the p-Nitroanilide Assay

For these determinations, the assay protocol outlined above was used except that the final substrate concentrations were changed and had values between 0 and $426\mu\text{M}$. The rates were determined as before in a total assay volume of $600\mu\text{l}$ using the prepared DAPI ($5\mu\text{l}$, 1u), and carried out at 37°C .

A double reciprocal plot of the Lineweaver-Burk type was made and the K_m and V_{max} values determined for DAPI using gly-arg-pNA as substrate under the conditions described. (see Results section 2.4.2.3).

2.3.3 Characterization of DAPI

2.3.3.1 Molecular Weight Estimation

(a) TSK-HPLC Method

This estimation method is based upon a specific elution time (under fixed - flow rates) from a protein specific separating HPLC column (TSK). The column separates according to molecular weight, much akin to the much used gel-filtration media 'soft' columns. The technique provides a rapid means of molecular weight determination but can suffer from a few problems. If at all possible the molecular weight marker proteins and sample protein must be as physically and chemically similar as possible to avoid other factors eg. shape (globular, or fibrous) having an effect on elution.

The following protocol was employed to give an estimation of the molecular weight of undissociated DAPI;-

- (a) The TSK-column was equilibrated with phosphate buffer (50mM, pH7) containing 2-mercaptoethanol (2mM). The flow rate was set at 0.6mls/min, pressure at 130psi and detection obtained on an absorbance detector set at 280nm.
- (b) Standard protein samples (2.1mg/ml) were made-up in the equilibration buffer, and the samples filtered. The standard proteins used and their molecular weights are listed below;-
Cytochrome C (12.5kda), Chymotrypsinogen A (25kd), Albumin (Hen Egg, 45kd), Albumin (Bovine Serum 68kd), Aldolase (150kd), Catalase (240kd) and Ferritin (450kd). Blue dextran (2000kda) was also applied and was used to determine the void volume of the

column, because, it was assumed, being so large, elution would take place with the 'solvent' front. (The void volume calculated value also included the dead-volume of the apparatus and connecting tubes).

Furthermore, sodium azide (0.1%) was also applied to the column and was used as a means of identifying where the last whole peak would elute from the column (because of its small size), after which any peaks appearing were attributed to solvent and protein sample impurities.

(b) PAGE Determination

The second molecular weight estimation method of DAPI was carried-out by the use of a non-dissociating, discontinuous polyacrylamide gel (vertical), using molecular weight standards (As used in section 2.3.3.1(a)) and stained, after electrophoresis with Coomassie Brilliant Blue R250 protein stain. The gel consisted of a resolving gel (7.5% polyacrylamide) with a stacker gel (4% polyacrylamide) placed on top, for ease of loading and to take advantage of the stacking effect caused by this discontinuous system. The following protocol was set-up for the gel construction.

COMPONENT	GEL (30 ml) (7.5%)	RESOLVING GEL (30 ml) (4%)	STACKING GEL (30 ml) (4%)
ACRYLAMIDE:BISACRYLAMIDE 30:0.8	7.5 ml		4.0 ml
GEL BUFFER	3.75 ml*		3.75 ml ‡
AMMONIUM PERSULPHATE (1.5%)	1.5 ml		1.5 ml
ddH ₂ O	17.25 ml		20.75ml
TEMED	20µl		20µl
(When ready to pour)			

*Resolving Gel Buffer - TRIS/HCl (0.6M, pH7.5) Titrated using 0.1N HCl.

‡Stacking Gel Buffer - TRIS/HCl (0.5M, pH6.8) Titrated using 0.1N HCl.

Gel Dimensions - Stacking Gel: 15x8x0.2cm (+ Comb)
Resolving Gel: 15x10x0.2cm

When the gel components (minus TEMED) were mixed the solutions were degassed for 2min. Upon addition of TEMED the gels were quickly poured and allowed to set at room temperature (17°C). After pouring the resolving gel, a thin layer of n-butanol was carefully layered on top to prevent air re-dissolving into the gel. Sample application wells were formed by the insertion of a teflon comb consisting of 11x0.9cm wide teeth into the poured stacking-gel. This gave sample wells volumes in excess of 100µl.

Molecular weight standards (Cytochrome C (12.5kd)) to Ferritin (450kd) as used in section 2.3.3.1(a) were used, one per well. (50 μ l; 1mg/ml). Samples of purified and unpurified (Post and Pre-HPLC purification steps) were prepared (as were the standards) by mixing the aqueous samples with a glycerol:bromophenol-blue:water (60%:0.5%:40%) solution in a 1:1(^w/v) ratio. Aliquots of each sample (enzyme) between 20-90 μ g were added to known wells.

Electrophoresis was carried-out at normal polarity and 6mA constant current, on a Volkam power supply, for 15hrs at room temperature (17°C). The reservoir buffer used was a TRIS/Glycine buffer. (0.186M Glycine in aq. 25mM TRIS, pH8.3). The gel was stained with Coomassie Brilliant Blue R250 (0.1%) in ddH₂O:methanol:glacial acetic acid (5:5:2) after filtering through a Whatman No 1 paper, for 5hrs. Destaining was achieved using the staining solution minus the stain. Visualization of the blue-stained protein bands was carried-out under normal light.

2.3.3.2 Isoelectric Point Determination of DAPI

The IEP determination of DAPI was carried-out using the following protocol, by disc-gel electrophoresis. A polyacrylamide gel (8%) was made using acrylamide:bisacrylamide (20:0.8, 10mls), ddH₂O (14.75mls), ampholines (pI range 3-10, 0.26mls), ammonium persulphate (1.5mls of a 1.5% ^w/v solution) and just prior to pouring TEMED (15 μ l).

The solution was degassed prior to TEMED addition, and the glass tubes to be used (10x0.6cm) were first washed in strong alkali

(1.0N NaOH) to avoid gel-swelling and wall adherence problems by maximizing the glass-surface polarity. One end of each tube was stoppered and the gels poured. (2.5mls per tube) and sealed with an overlay of ddH_2O . Polymerization was achieved overnight (Δ 15hrs) at 18°C . After addition of the samples (preparation outlined below) the gels were run on a disc-rod gel electrophoresis apparatus at 5V/cm (40V total) for 2hrs and then 15V/cm (120V total) overnight (15hrs) at 18°C . The analyte solution used was 0.2% sulphuric acid plus ampholine solutions (1%, pI 3-10) and the catholyte solution was 0.1% diethylamine amine plus ampholines (1%, pI 3-10).

The samples, in glycerol (20% v/v) were loaded at the anode end of the gels. For each of the following samples duplicates were run:- purified DAPI (Post HPLC step, 30 μg /tube), unpurified DAPI. (Pre HPLC Step, 580 μg /tube) and commercially available DAPI from Boehringer (In 50% glycerol, 20 μg /tube), all loaded as 20 μl samples. The last two gels were run as blanks and to provide a means of determining the pH-gradient down the gels for calibration curve construction.

After focusing the gels were removed using a long hypodermic needle and ddH_2O straight into boiling tubes containing Coomassie Brilliant Blue Stain (As for section 2.3.3.1). Staining was carried-out for 12hrs at 18°C with destaining for 48hrs at 18°C . Visualization of the protein bands was under normal light. A pH/pI-calibration curve was determined by cutting the two blank gels (separately) into 0.5cm portions and mixing thoroughly in 0.5ml ddH_2O portions to leech out the ampholines. (These two gels do not undergo staining). The pH of each sample

was then determined using limited range pH papers from E.Merck, F R Germany and a calibration curve of pH vs distance migrated from the anode (cm) was constructed. Approximate pI values were then assigned to the stained sample gels.

2.3.3.3 Specific Activity Calculations

Specific activity calculations give a means of purification factor calculation ie. monitoring of the increases in purification between stages and also give an indication of the 'potency' of the enzyme preparation. Specific activity is defined as the number of enzymic units per mg of protein. One unit is defined as one μ mole of substrate hydrolyzed or product formed/min under fixed conditions and for a named substrate, since specific activity varies from substrate to substrate and with changing conditions (ie. temperature, substrate, concentration, pH etc).

Therefore, an appropriate enzyme assay (see section 2.3.2) and protein concentration assays are required for specific activity calculations to be carried-out.

For DAPI the specific activity of the preparation and final enzyme samples were calculated and a table of the results can be seen in section 2.4.2.1, and analyzed, in part, in section 2.4.3.3.

2.3.3.4 Storage Tests

It was useful to determine the stability of this enzyme under recorded conditions and storage in a bid to optimize those storage conditions for maximal retained activities. Some work upon this is detailed in Chapter 3, sections 3.3.2, 3.3.3 and 3.3.4.

Enzyme solution aliquots both pre and post HPLC stage purified (section 2.2.1) were subjected to one of the following:-

- (a) Storage at -20°C , $+4^{\circ}\text{C}$ and 18°C for the same time, and underseal and;
- (b) lyophilization and subsequent re-suspension and
- (c) stored at 4°C but open to the air. (To test for the effects of sulphydryl reagent oxidation upon enzymic activity loss).

The samples were then assayed for activity as described in section 2.3.2 and the results compared and discussed in section 2.4.3.4.

2.4 Results and Discussion

2.4.1 Purification of DAPI

The purification of DAPI was largely based upon the method of Mettrione et al with the TSK-HELIC added. This method gave a preparation of DAPI of apparent homogeneity (see Fig. 2.4.1 (c), Re-chromatography). The preparation, along with purchased "standard" enzyme was used in all of the characterization methods analyzed, and also for the immobilization studies (Chapter 3).

The method outlined produced a high specifically active preparation quite rapidly and was highly reproducible. A purification of 81x was achieved with an overall recovery of approximately 5%.

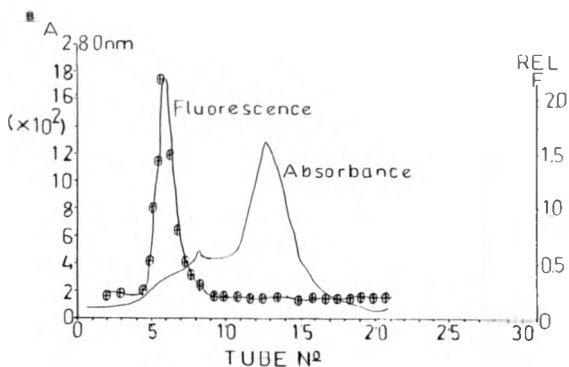
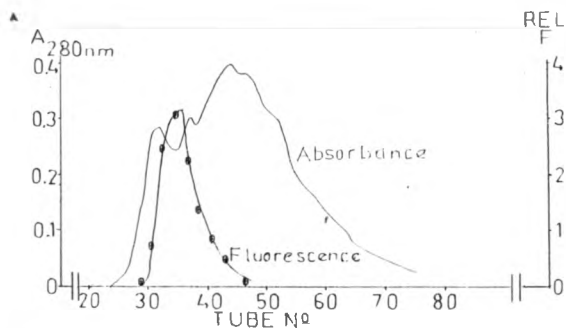


Fig. 2.4.1.(a) DAPI Elution following Sephacryl S-300 Gel Filtration Chromatography

Fig. 2.4.1.(b) DAPI Elution following TSK-HPLC Chromatography

(—) Absorbance profile at 280nm corresponding to protein elution and
 (●●) Relative fluorescent enzymic activity present in the eluted protein profile

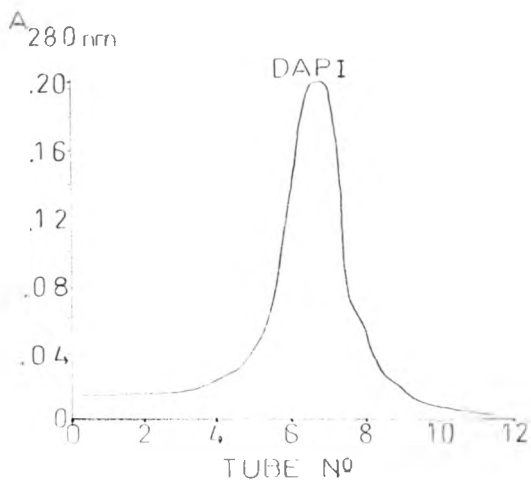


Fig. 2.4.1 (c) DAPI TSK-HPLC Re-chromatography

Table 2.4.1 Purification of Diaminopeptidase I (DAP)

CATEGORY	VOLUME (mls)	PROTEIN CONCENTRATION (mg/ml)	TOTAL UNITS (U)	SPECIFIC ACTIVITY (U/mg P)	PURIFICATION FACTOR (X)
SlICE					
POST-EXTRACTION & HOMOGENIZATION 930 (ACIDOLYSIS / CENTRIFUGATION)	1,000	107.0	161 KU	1.51	1.00*
		12.0	44.3 KU	3.97	2.6
POST AMMONIUM SULPHATE FRACTION ^N (40-70% CUT) HEAT TREATED/ ULTRAFILTRATION	1060 (S/N)	5.0	1,070	0.201	-
	100	39.0	34,050	8.73	5.82
POST-GEL FILTRATION (S300, SEPHACRYL)	20	29.0	29,000	50.0	33.1
POST TSK-HPLC	5	12.0	7,380	123.0	81.4

(* Referred to as basal quantity)

S/N N Supernatant
Frac Fractionation

2.4.2 DAPI Assays

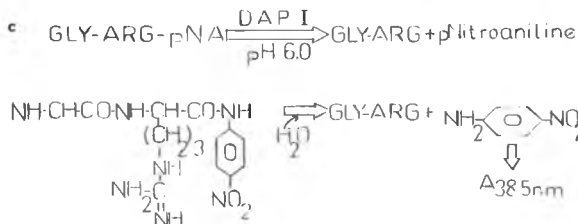
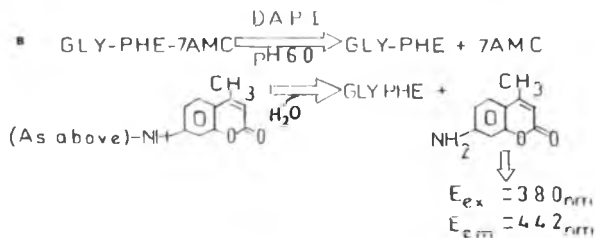
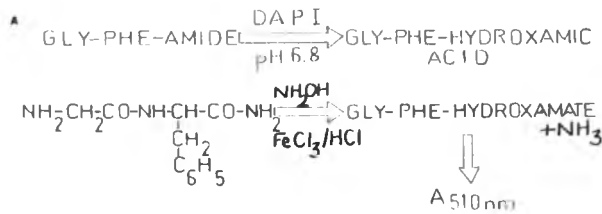
2.4.2.1 DAPI Transferase Assay

It was reported by de la Haba et al (Ref. 39), that DAPI has a transferase activity at pH6.8, the pH used for this assay. The assay's progression is monitored by the spectrophotometric measurement of the dipeptide product when transferred to hydroxylamine to give a dipeptidyl (Glycyl-L-phenylalanyl) hydroxamic acid derivative (Fig. 2.4.2.1 (a)). This assay was carried-out with different amounts of enzyme added under the conditions described. The responses obtained (Fig. 2.4.2.1 (b)) were linear for the first 15 min after enzyme addition, the maximal response for the assay with most enzyme added took up to 100 min to achieve.

As can be seen, the assay is linear over the early times of the assay and using the amounts of enzyme quoted, but the actual response is somewhat lower and hence comparably slower to those responses noted for other DAPI assays studied here.

2.4.2.2 Fluorescence Assay

The reaction involved in this assay is outlined in Fig. 2.4.2.2. It relies upon the excitation of a fluorescent group when detached from a dipeptidyl side group with subsequent monitoring of its emission at 442 nm. The group used in this assay is the Aminomethyl coumarin (AMC) group and it is initially excited at 380 nm. The assay depends upon the peptidase activity of DAPI which cleaves the amide bond between the amino terminal dipeptide (Glycyl-L-phenylalanyl, Gly-L-phe) and the AMC



A Fig 2.4.2.1 (a) DAPI Transferase Assay Reaction Scheme

B Fig. 2.4.2.2 (a) DAPI Fluorescence Assay Reaction Scheme

C Fig. 2.4.2.3 (a) DAPI p-Nitroaniline Assay Reaction Scheme

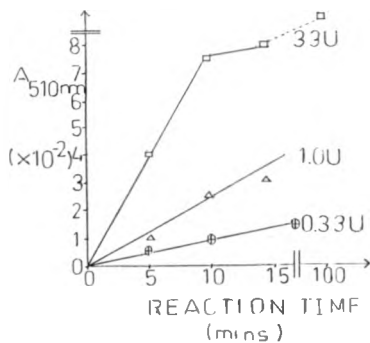


Fig. 2.4.2.1 (b) DAPI Transference Assay, Plot of Rate Dependence Upon Enzyme Concentration

group, which does not exhibit fluorescent properties when covalently bound.

The assay is quite sensitive and reproducible. As with most fluorescent assays, quenching may play an important part, especially if the substrate concentration is high. The advantages of such an assay include the provision of corroborative evidence for the presence of DAPI along with the p-nitroanilide assay discussed in section 2.4.2.3. Prior to the use of Gly-L-phe-7AMC as a substrate, a second fluorescence assay utilizing the release of β -naphthylamine was widely used. McDonald *et al* describe the procedure as rapid, convenient and reliable, as it appears to be. However, since the publication of their paper the naphthylamides are not now widely used because of their known carcinogenic properties. They quote a sensitivity factor for their fluorescence assay over the dipeptidyl transferase assay of up to one hundred times. Comparisons with the similar assays studied herein underline this fact to some extent; when the rates of activity become significant and easy to monitor then the assay was deemed to be detecting the enzyme. Far less enzyme and substrate are needed for the fluorescence assays than for the transferase assay. A second assay studied utilizes the same type of reaction of DAPI as for the fluorescence assay and is characterized by the production of a non-fluorometric but spectroscopically detectable product, p-nitroaniline (section 2.4.2.3; below). Thus, a study of this assay may 'mimic', and indeed did, the trends observed for the fluorescence assay. From direct and double reciprocal plots (Fig. 2.4.22 (b) and (c) respectively) the K_m -value of Gly-L-phe 7AMC under the conditions described was calculated to be 0.33 ± 0.05 mM and the maximal velocity V_{max} to be 0.66 ± 0.05 mM/min.

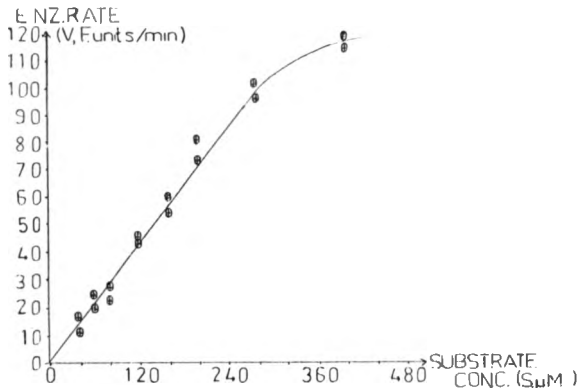


Fig. 2.4.2.2 (b) Direct Plot of DAPI Enzyme Rate versus Gly-Phe-7AMC Concentration

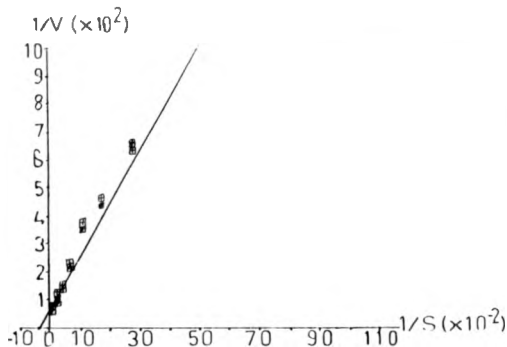


Fig. 2.4.2.2 (c) Double Reciprocal Plot of DAPI Enzyme Rate versus Gly-Phe-7AMC Concentration

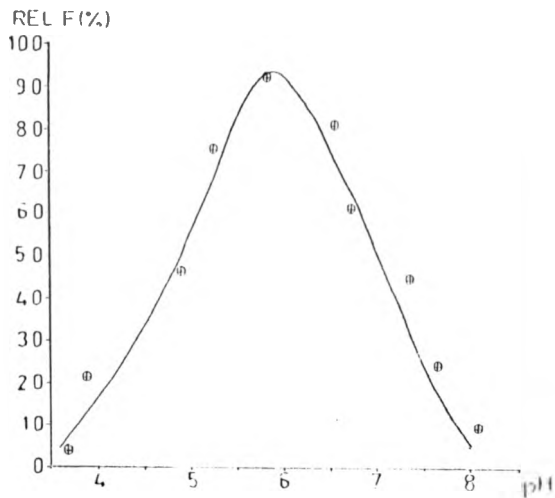


Fig. 2.4.2.2 (d) pH-Profile of Fluorescence Assay of DAPI

The pH-dependence of the Gly-L-phe 7AHC assay with DAPI was also studied. Once again, the profile (Fig. 2.4.2.2. (d)) was similar to that reported for the β -naphthylamide derivative hydrolyses by McDonald et al (1969) (Ref. 31). Both profiles had pH-maxima at around pH6, but curiously the profile for the Glycyl-L-phenyl- β -naphthylamide assay exhibits two peaks around pH6.0, but the profile one obtained for the fluorescence assay detailed here exhibits only one. The differences are hard to define except to point out that the inflexion point between the two peaks is shallow and may be attributable to artefactual properties of the assay or measurements.

2.4.2.3 p-Nitroanilide Assay

The mechanism of the reaction is outlined in Fig. 2.4.2.3 (a). A direct plot of enzymic rate against varying substrate concentration shows a classic Michaelis-Menten curve (Fig. 2.4.2.3 (b)). The assay was found to be more reproducible and simpler to perform when compared to the transferase and fluorescence assays outlined earlier.

A double-reciprocal plot (Lineweaver-Burk) of these results give the equilibrium constant, K_m and the maximal velocity, v_{max} of the assay. These values were calculated to be $1 \pm 0.05mM$ and $0.083 \pm 0.02 A A405 nm/min$ respectively. These simple one-step assays utilizing p-nitroaniline formation are now used quite widely for peptidase-type assay as is illustrated later on in this study for the assay of Acetylaminopeptidase (Chapter 4).

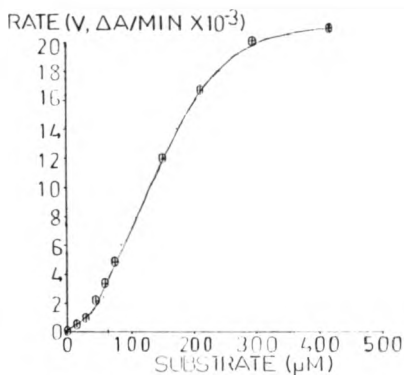


Fig. 2.4.2.3 (b) Direct Plot of DAPI Enzyme Rate versus GlyArg-pNA Concentration

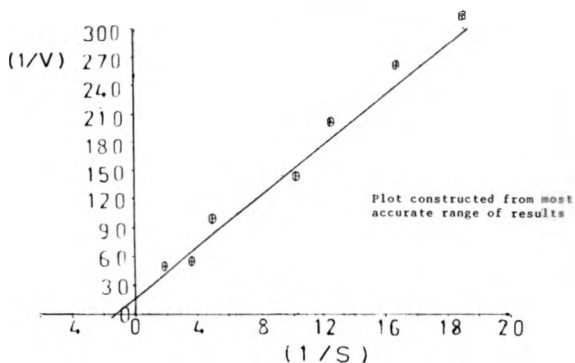


Fig. 2.4.2.3 (c) Double Reciprocal Plot of DAPI Enzyme Rate versus GlyArgpNA Concentration

2.4.3 Characterization of DAPI

2.4.3.1 (a) TSK-HPLC Determination of the Molecular Weight of DAPI

The results of the HPLC determination of DAPI's molecular weight are shown as a direct plot of corrected elution time (min) against the molecular weight of the components (K daltons) (Table 2.4.3.1 (a) and Fig. 2.4.3.1 (a)). The molecular weight of DAPI was determined from the calibration curve and was shown to be 190 ± 10 K daltons. This value closely resembles those quoted in former publications.

Thus, this method of molecular weight determination appears to be valid for DAPI and does not suffer from any of the inherent problems postulated earlier. For accurate analysis and corroborative evidence, a second method of molecular weight determination was carried out and is detailed in the following section (section 2.4.3.1 (b)).

2.4.3.1 (b) PAGE Estimation of the Molecular Weight of DAPI

A calibration curve of the molecular weights of the marker proteins used as standards against their mobility down the gel was used to determine the molecular weight of DAPI by direct comparison. The calibration curve constructed is shown in Fig. 2.4.3.1. (b).

The molecular weight of DAPI was calculated to be 210 ± 20 Kdaltons, a figure in good agreement to that calculated in section 2.4.3.1 (a). Both values obtained are very similar to the value obtained by Planta and Gruher (Ref.33) i.e. 210 ± 30 Kdaltons.

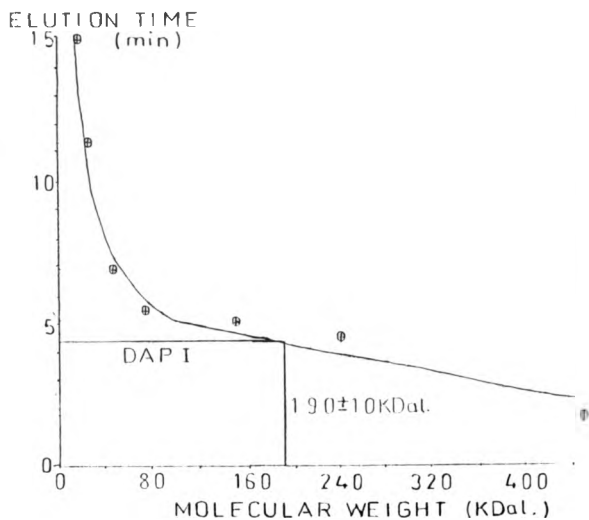


Fig. 2.4.3.1 (a) TSK HPLC Molecular Weight Determination of DAPI

Table 2.4.3.1.(a) Elution Times of Molecular Weight Standard Proteins

Protein	Molecular Weight (K dal)	Corrected Elution Time (min)
Cytochrome C	12.5	15.0
Chymotrypsinogen A	25.0	11.3
Hen's Egg Albumin	45.0	7.0
Bovine Serum Albumin	68.0	5.5
Aldolase	150.0	5.0
Catalase	240.0	4.5
Ferritin	450.0	1.8

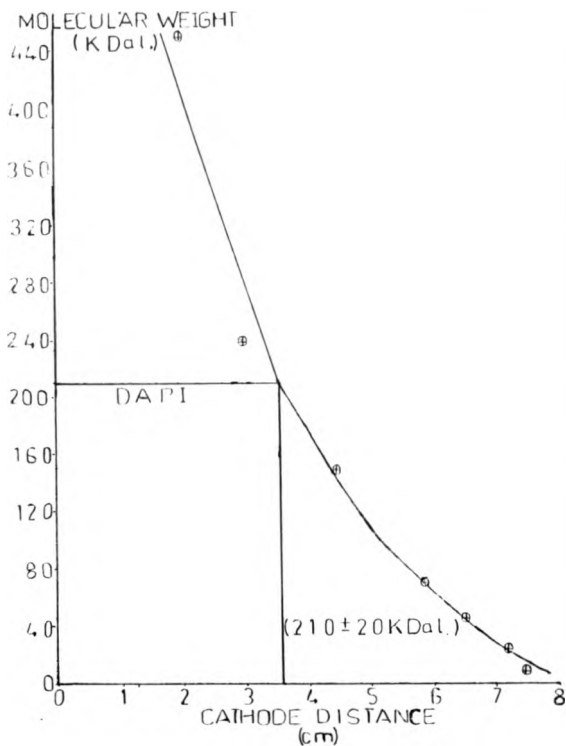


Fig. 2.4.3.1 (b) PAGElectrophoresis Molecular Weight Determination of DAPI

2.4.3.2 Isoelectric Point (IEP) Determination of DAPI

Isoelectric point determination of proteins is a very important technique used for the characterization of enzymes. It can also aid the formulation of improved separation and purification procedures of a particular protein (eg. preparative isoelectric focussing). The technique is based upon the hypothesis that the isoelectric point of a protein is the pH at which the net charge of a protein is zero and is a direct function of the amino acid composition. This point, when attained means that when placed in a pH gradient under fixed conditions the protein molecules would migrate to, and be held at, a position in the gradient corresponding to its isoelectric point. Any deviations from that point (eg. by diffusion) would result in a charging of the protein molecule, either positively or negatively, which would then cause a re-focussing back to its original position. Thus, in general, less protein is needed than in other electrophoretic techniques because of the tighter banding observed in isoelectric focussing. The isoelectric point of a protein is not totally unique to a given protein, but the evaluation of it is a useful piece of information to obtain for its characterization and identification, and together with other analyses provide a unique 'description' of the protein studied.

In addition to this, the technique gives one a means of assessing the purity of the sample tested in a similar way to that observed in a PAGE analysis (ie Molecular weight determination section 2.4.3.1 (b)) and TSK-HPLC profile (section 2.4.3.1 (a)).

One method of isoelectric point determination is by the use of Ampholines, a range of molecules with different isoelectric points, between a required range (eg. pI 3-10) which upon focussing, form a pH-gradient across a gel. Samples added would migrate down the gel until it reaches the position in the gel equivalent to its own isoelectric point. Identification of a particular IEP can be achieved by the construction of a standard curve from a known group of proteins migration distance from an electrode and their corresponding isoelectric points; and the subsequent determination of an unknown samples IEP by reference to the curve after its migration distance has been determined. Alternatively, the pH of the focussed gel at various points down the gel can be determined by a point electrode or by the elucidation of the pH of gel slices (perpendicular to the applied field) by dissolution into known volumes of deionized water with subsequent pH determination by a micro-electrode or pH papers.

A second method of isoelectric point determination is utilized in the characterization of Acetylaminopeptidase which is detailed in Chapter 4 of this study. This method uses a flat-bed pre-formed IEP gradient gel and standard isoelectric point proteins.

The isoelectric point of DAPI was found to be 5.2 ± 0.2 , determined by direct comparison to the standard isoelectric point curve constructed and shown as Fig. 2.4.3.2. The distance migrated by the purified DAPI was measured and the corresponding pI-value read off. This value was found to be quite close to that observed for the sample purchased from Boehringer, which, in turn was close to that reported by McDonald et al (Ref 31).

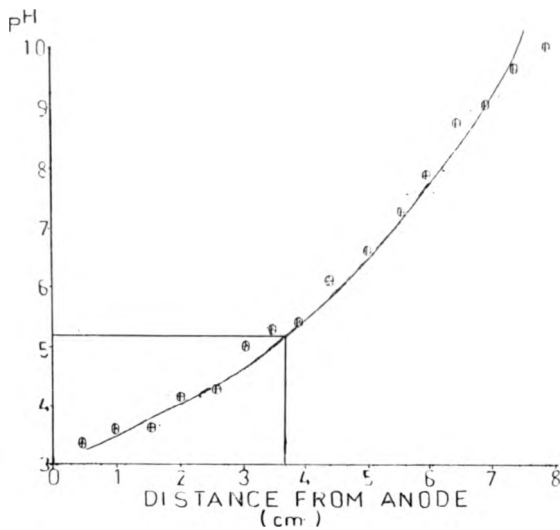


Fig. 2.4.3.2 Isoelectric Point Determination of DAPI

This method of isoelectric point determination has major disadvantages when one compares it to that pI-determination method utilized in Chapter 4. Firstly, the time involved in the determination is far greater, due to longer periods required for polymerization, staining and destaining. Secondly, the estimation of the pH of each gel segment depends upon the accuracy of the pH-electrode or papers used, and the efficiency of the 'leaching-out' of the ampholines for the IEP determination to occur. Lastly, standard value IEP-protein kits could also be employed (As in Chapter 4) which would provide more accurate determinations - because these protein bands obtained after electrophoresis would have been subjected to the same conditions as the sample and are more representative of the way in which a protein would act under the conditions described and the IEP determination would not solely rely upon the pH-determination of each gel-segment, with all the inherent faults this may entail.

2.4.3.3 Specific Activity Calculations

The specific activity of the enzyme samples analyzed are calculated from the enzymic rate with a substrate and the protein concentration of that sample. In DAPI's case the specific activity values quoted in table 2.4.1 are based upon the fluorescence assay detailed in section 2.3.2.2. and the Hartree modification of the Lowry protein estimation assay. The actual values are expressed as the amount of enzyme (1 unit) required to hydrolyze 1 μ mole of Glycyl-L-phenylalanyl 7AMC per min under the given conditions, and the specific activity values as the number of units/mg of protein.

2.4.3.4 Storage Tests

(a) Temperature Effects

A sample of pre- and post-HPLC purified DAPI was incubated at -20°C for 24 hours and then assayed. The samples appeared to be quite stable when frozen but only exhibited an activity of 90% (Pre-HPLC) and 93% (Post-HPLC) of those values exhibited prior to freezing. Thus, a certain amount had become denatured and it has been postulated that DAPI does not like to be frozen in the presence of 2-mercaptoethanol.

Second samples of enzyme were stored in a fridge at 4°C for 24 hours and when assayed most of the activity (96-98% for both samples) was detectable.

Lastly, third samples were allowed to stand at 18°C for 24 hours and then assayed. Large losses of activity were noticed after incubation. Only 22% of the Pre-HPLC sample and 36% of the Post-HPLC sample's activities remained. The difference between the two could be attributable to contaminant protease-type enzymes which would accelerate enzymic loss by degradation.

Thus, one could conclude that storage of a solution of DAPI at pH7 in the presence of 2-mercaptoethanol appears to be preferable at 4°C.

(b) Lyophilization

Only post-HPLC enzyme was subjected to lyophilization (or freeze drying)

immediately after purification. Upon reconstitution in the same volume as lyophilized, the percentage retained activity had dropped to 70%. Losses upon freeze drying have been reported before, but the loss in this case may have been accentuated by the presence of 2-mercaptoethanol, as in the temperature effects upon storage section above.

(c) Storage at 4°C (sample open to air)

The samples used contained the same amount of 2-mercaptoethanol (2mM), but were stored at different pH's i.e. pH 4.5 for the Pre-HPLC sample and pH7 for the post-HPLC sample. This difference may play a part in the stability of the enzyme when stored at any temperature (see earlier) especially if oxidation of the sulphhydryl reagent, is important. When left open to the air the samples were subjected to air oxidation of 2-mercaptoethanol. However, the samples were not constantly mixed and the samples stored sealed at 4°C also had air trapped in the bottles. After storage for 2 weeks the open samples were assayed. The pre-HPLC sample exhibited only 80% enzyme activity retention and the post-HPLC sample exhibited 85% retention of activity. Without, further study it could not be determined whether the actual losses were due to oxidation, pH-difference, protease contamination, enzyme concentration or combinations thereof. Suffice it to note that DAPI samples are best stored in solution, sealed, at 4°C and as pure as possible, according to the results detailed above.

CHAPTER 3

Immobilization of Diaminopeptidase I (DAP I) (E.C.3.4.14.1)

Section 3.1 Introduction

Many research projects and biological processes are based entirely on the ability to create the natural systems for study. There are inherent problems with this, relating on the whole, to the system's components becoming unstable and losing their biological activities. Furthermore, economically orientated reasons such as, component cost, scarcity or difficulties in preparation are very important especially in industry. Therefore, a method for retaining a component's viability for multiple use is a big step towards surmounting the above problems. Such a method can be developed utilizing the technique of immobilization. Several such systems have been developed which are designed to alter an enzymes' stability kinetic parameters, and substrate specificities, which not only mimic the natural systems, but also improve upon them for increased productivity, making them ideal for commercialization.

The adsorption of an enzyme onto an insoluble support is the simplest method of immobilization. Basically, it consists of incubating the support material and enzyme for a stated time and appropriate conditions, whereafter the adsorbed enzyme-support is separated from any excess soluble enzyme by centrifugation or filtration. The immobilized enzyme can then be formed into a column and substrates added and slowly eluted through with subsequent products collected at the end. The major disadvantage of this method is that the enzyme is not firmly bound. Changes in the pH- or salt concentration of solutions used can drastically

alter adsorption. However, this immobilization method is especially useful for multimeric enzymes where covalent and harsher methods may alter activities and any possible allosteric effects present.

Covalent coupling for the immobilization of enzymes is based upon the formation of covalent bonds between the enzyme and support material. Obviously, if covalent bonding occurs between the support and active site amino-acids this will alter their enzymic activities. However, if immobilization takes place in the presence of its substrate, which tends to protect the active site, then this problem is largely overcome.

Activated supports target their attack particularly upon α - and ϵ -groups of lysine, tyrosine, histidine, arginine and cysteine residues. Such supports, upon activation, used include sepharose, cellulose and polyacrylamide co-polymers. Advantages of this method include firm binding of enzyme, which avoids leakage, under various conditions, and its application to enzyme purification by the use of a covalently bound affinity label for the enzyme and the inert support.

A third most common method of immobilization is gel entrapment of entire cells and enzymes. The previous two methods are not always useful because of the reasons mentioned and also because of the physical reactions involved which can alter the diffusion of substrate deleteriously and hence activity. Gel entrapment can immobilise whole cells, which requires less covalent cross-linking and also provides larger areas for adsorption and interaction. This technique can be used to catalyse whole pathways from, for example, glucose to ethanol via several steps. Furthermore, highly purified enzymes are not needed for this method, although this has obvious limitations on reaction control and accuracy.

An application of the use of immobilization for enzymic analysis is detailed in this chapter including an example of the use of such a system to hydrolyse a pentameric peptide into component parts.

Commercial and purified preparations of Diaminopeptidase I (DAPI) (Detailed in Chapter 2) were immobilized to a variety of alkyl- or aryl-Sepharoses by hydrophobic binding, and to cyanogen-bromide-activated Sepharose by covalent binding. This was carried out in a bid to form stable preparations of DAPI, and to use them in the analysis of peptidyl sequences and/or N-terminal amino acid sequences of proteins. The advantages of immobilized over unbound native enzyme preparations when used in sequence analysis, include the ease of separation of digestion products when no free enzyme moiety is present to interfere, and the fact that in some cases the time course of the reaction is sufficiently slowed so as to enable the identification of the first, second etc digestion products to be determined and hence sequence. Steric effects are postulated as having an effect upon the speed of reaction of immobilized enzyme preparations.

This chapter includes the preparation of immobilized DAPI to alkyl- or aryl-Sepharoses (Fig. 3.4.1) by hydrophobic bonding, cyanogen-bromide Sepharose by covalent binding, and their applications to peptidyl sequence analysis with subsequent identification of products by reverse phase HPLC. The optimum pH of the immobilized enzyme, its stability to repeated use and stability to temperature changes are all compared to preparations of soluble DAPI.

Section 3.2 Materials

3.2.1 Sources of Diaminopeptidase I (DAPI)

Commercial DAPI was purchased as a 50% suspension in glycerol from Boehringer Corporation, London Ltd. Purified DAPI (As purified in Chapter 2) in sodium acetate (0.1M) buffer containing sodium chloride (0.1M) and 2-mercaptoethanol (2mM), pH4.5.

3.2.2 Immobilization Supports

The "C_n"-series of alkyl agaroses (where n represents the number of carbon atoms in an alkyl chain attached to the agarose) was obtained from Miles Scientific (Slough UK).

Octyl- and phenyl-sepharoses and cyanogen-bromide-activated sepharose were obtained from Pharmacia (Milton Keynes, UK).

3.2.2 Assay Materials

7-Glycylphenylalaninamido-4-methylcoumarin (7-AMC) was purchased from Universal Biologicals (London, UK).

The acetate salt of glycylarginyl p-nitroanilide (GlyArg-pNA) was purchased from Sigma Chemicals Ltd (Poole, UK).

Section 3.3 Experimental

3.3.1 Hydrophobic Immobilization

A series of columns (6 x 1ml capacity) of Cn-agaroses (n=0,2,4,6,8 or 10) were washed with potassium phosphate buffer (50mM) containing 2-mercaptoethanol (12mM) and sodium chloride (1% w/v) (20ml, pH6.7). A sample of prepared DAPI (50 μ l \approx 1 unit) was added to each column and allowed to interact with the support for 10 min. at 17°C. The columns were then washed with the above buffer (2 x 2ml) and the eluates assayed for unbound enzyme using the substrate. Gly-Arg-pNA (By the method detailed in Section 3.3.5.1). The percentage activity found in the eluates were then obtained. It is worth noting at this point that the 2 x 2ml buffer washings per column were found to be sufficient to elute the great majority of unbound enzyme. Only negligible amounts of enzyme were eluted by further washings.

3.3.2 Enzyme Stability

Columns (1ml) of DAPI immobilized as described in the preceding section, to phenyl- or octyl-Sepharose were prepared and the activity of the bound enzyme determined. Repeat enzyme activity assays of both columns were carried out after 10 min., 1 hr, 24 hr, 48 hr and 96 hrs. The columns were incubated at 4°C between assays, but were warmed to room temperature (Ca 17°C) prior to assay.

3.3.3 Thermal Stability

Di-aminopeptidase I (2U) was immobilized onto octyl-Sepharose (8ml) previously equilibrated with the buffer used in Section 3.3.1.

The batch was then divided into eight equal aliquots of 1ml each. The aliquots were incubated at different temperatures for 10 min., cooled to room temperature (17°C) and then assayed for retained activity. For comparison, soluble enzyme aliquots (20µl of 2U/ml solution) were incubated at the same temperatures above and the retained enzyme activity assayed.

3.3.4 pH-Stability Profile

Phosphate buffers at ten different pH-values between 4 and 8, each containing 2-mercaptoethanol (12mM) and sodium chloride (1%, w/v) were prepared. Ten columns of octyl-Sepharose (1ml) were prepared with the commercial DAPI preparation (0.02U, 10µl) added to each column and each equilibrated with one of the prepared buffers. Each column was then incubated at 17°C for 15 min., washed with the same buffer, and the activity of the bound enzyme determined. The substrate, Gly-Arg-pNA was stable over this pH-range, and the absorption of a known concentration of p-nitroaniline was shown not to alter significantly over this pH range. For comparison, DAPI (0.01U, 10 µl) was each added to the ten different pH-buffers (600µl) and incubated at 17°C for 10 min. The enzyme activities were then determined.

3.3.5 Enzyme Assays

3.3.5.1 Soluble DAPI

For each sample of enzyme to be assayed, the substrate GlyArgpNA (10µl, 9.7mM) was added, the solution was then incubated at the required temperature, pH and time and the enzyme activity determined by the change in absorbance of the solution at 385nm.

3.3.5.2 Immobilized DAPI

The enzyme samples immobilized on phenyl- or octyl- Sepharose (1ml bed volumes) were mixed with the substrate, Gly-Arg-pNA, (10 μ l, 9.7mM) and the reaction allowed to proceed under the conditions of temperature, time and pH as required.

The immobilized enzyme was washed with a known volume of buffer and the optical absorbance at 385nm determined. Initial experiments showed that all of the p-nitroaniline formed was eluted from the columns by the volume of buffer stated. The enzyme activities of soluble DAPI preparations were checked using the fluorescence assay described in Chapter 2, and the protein concentrations of preparations determined using the Hartree modification (Ref.59) of the Lowry protein estimation assay. (Ref. 67).

3.3.6 Digestion of Methionine-Enkephalin by Immobilized DAPI

DAPI(3 μ) was immobilized onto octyl-Sepharose (2ml bed volume) and this was divided into two equal portions. Each portion was washed in a centrifugation tube with 10x1ml incubation buffer (50mM potassium phosphate containing 12mM 2-mercaptoethanol and (40% w/v) sodium chloride, pH5.8). Methionine enkephalin (40 μ l in buffer, 15.5mM) was added to each portion and incubated at room temperature. At different time intervals, the incubated tubes were centrifuged at low speed and an aliquot (10 μ l) of the supernatant was subjected to analysis by reverse-phase HPLC. (Spherisorb ODS-10 column, (300x4.6mm) elution with 35% acetonitrile/65% demineralized water/0.1% trifluoroacetic acid, with detection at 214nm) to study the breakdown of the peptide. After each test the samples were gently agitated to ensure thorough remixing of the substrate with the immobilized enzyme.

3.3.7 Cyanogen Bromide Activated-Sepharose Immobilization

This procedure was largely based upon that described in the Pharmacia booklet, "Affinity Chromatography: Principles and Methods" (p.15, Ref.63). The following conditions were used where an alternative buffer and pH are allowed.

- (a) The enzyme and gel suspension was mixed in an end over end mixer overnight (Δ 16hrs) in the coupling buffer (NaHCO_3 , 0.1M, pH8.3 containing sodium chloride (0.5M).
- (b) The remaining active groups were blocked using glycine buffer (0.2M) for 2hrs at room temperature, this blocking agent was then removed (uncoupled) by further washing with coupling buffer.
- (c) The enzyme-Sepharose conjugate was stored at 4°C until used.
- (d) Any unbound protein was assayed by the Hartree protein assay. (Ref. 59) method.

Only negligible amounts of protein were detected in the supernatant above the settled enzyme-Sepharose conjugate, therefore, it was assumed that virtually all of the enzyme had bound in some form to the CNBr-activated Sepharose.

Section 3.4 Results and Discussion

3.4.1 Hydrophobic Immobilization

It was found that DAPI underwent hydrophobic binding to octyl- and decyl-Sepharoses very readily, and also to phenyl-Sepharose (see Fig. 3.4.1). It was convenient to use commercially available octyl-Sepharose as the support upon which the DAPI preparations are immobilized for the repeated assay, thermal and pH-stability studies. The length of the chain upon which DAPI ceased to bind ie $C_n(n=6-8)$ may give an insight into the depth within the enzyme at which one may locate the free group at which bonding takes place. At this stage one cannot discount the possibility that bonding takes place within the active site of one or more of the monomers making up the enzyme. Resultant loss in enzyme activity is difficult to ascertain, if this bonding takes place, because of the general trend that immobilized enzymes tend to have lower activities than their free-soluble moieties, and hence, the loss of activity may be due to the above case or to loss of active sites by other means. To determine which is the case would take detailed kinetic analysis, the study of which has not been entered into here.

3.4.2 Enzyme Stability (See Fig. 3.4.2)

The immobilized enzyme was stable when stored at 4°C, with over 50% of the original activity being retained after 4 days and 5 repeated assays. The rates of hydrolysis of GlyArgpNA by DAPI immobilized on octyl-Sepharose or phenyl-Sepharose gave V_0 (the maximum rate of

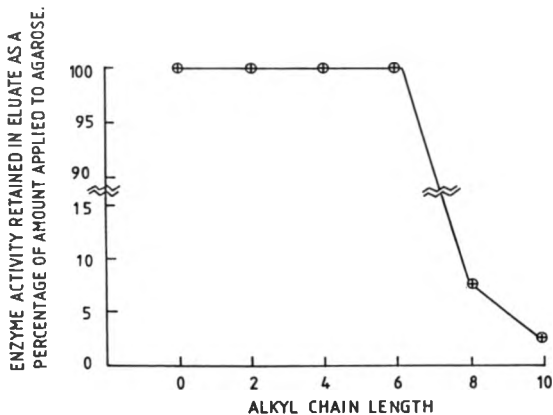


Fig. 3.4.1 Variation with Chain Length of Binding of DAPI to Alkyl Agaroses

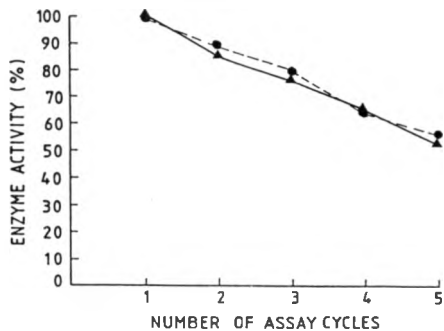


Fig. 3.4.2 Effect of Storage at 4°C on Activity of DAPI Immobilized on Phenyl- or Octyl-Sepharose

activity) values of 32% and 37% respectively of the V_0 value obtained for the soluble enzyme under identical conditions. Samples of DAPI immobilized on octyl- or phenyl-Sepharoses showed a slight drop in activity (approximately 20%) after 24hrs before use. After this time, no change in activity was observed after storage for up to 14 days before use. Repeated use of the same sample of immobilized DAPI caused a regular loss in activity with each assay. This finding was the same for octyl- or phenyl-Sepharose immobilized DAPI and is probably due to sequential loss of immobilized DAPI by successive washings prior to assay ie a form of leaching-out. Deactivation of the enzyme-support complex is not considered probable because of the evidence shown above.

3.4.3 Thermal Stability

The thermal stability of the soluble and octyl-Sepharose enzyme complex differs in an interesting way. It was found that the immobilized enzyme complex was more thermally unstable than the soluble enzyme. The results for the soluble enzyme are as expected, because one of the purification steps used included a heat treatment step up to 60°C for 40mins (Chapter 2), and so as one can see (Fig. 3.4.3) the activity of soluble enzyme is at a maximum at about 50-60°C, but thereafter there is a rapid decrease in retained activity up to 80°C. Normally immobilized-enzyme complexes are more thermally stable than the soluble enzyme,; but in this case it is not, and may be partially explained by previously hypothesized (see earlier) binding of the support to the active site and/or

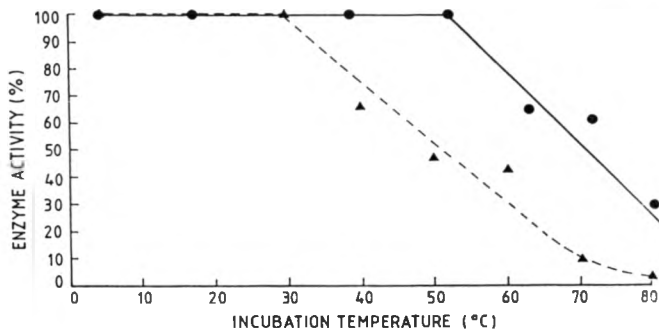


Fig. 3.4.3 Thermal stability of DAP I (—▲—▲—) immobilised on octyl-Sepharose, (—●—●—) soluble).

increased strain on the native oligomeric structure of the enzyme which may be exaggerated by temperature increases.

3.4.4 pH-Stability Profile

It was found that the pH optima of the soluble (pH5.8) and immobilized (pH 6.1) enzymes were very similar (Fig 3.4.4). Furthermore, these values are in the pH-optimum range reported by McDonald et al (Ref 31).

3.4.5 Enzyme Assays

The protocols used for the measurement of DAPI activity provided quick, relatively cheap and much safer means of assay than several methods previously used. Elution of the product of hydrolysis, p-nitroaniline was rapid and as a result an accurate representation of enzyme activity whilst immobilized was achieved.

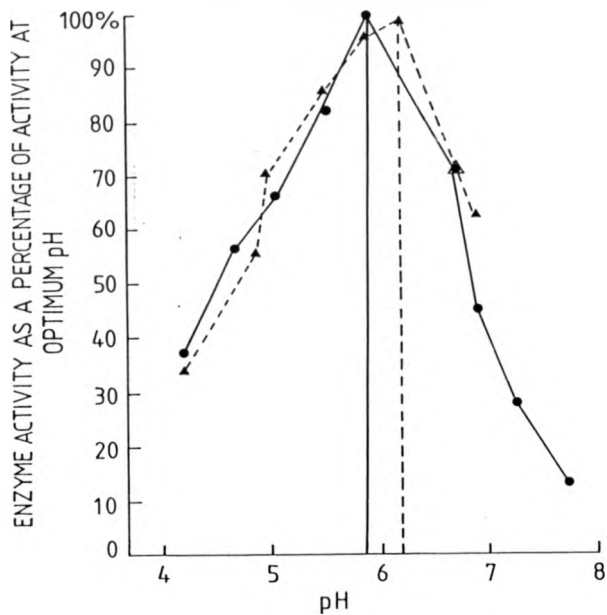


Fig. 3.4.4 Effect of pH on the activities of soluble (●—●) and immobilised (▲---▲) DAP I.

3.4.6 Digestion of Met-Enkephalin by Immobilized DAPI (see Fig. 3.4.6)

DAPI immobilized on octyl-Sepharose was capable of digesting, by hydrolysis, Methionine-Enkephalin (Tyr-Gly-Gly-Phe-Met) a pentameric bioactive peptide. The first products were detectable after 30min digestion at room temperature. The dipeptide, Tyr-Gly and the tripeptide Gly-Phe-Met were separable and identifiable by their elution times when compared to standard samples under identical conditions. After a further 50min digestion the tripeptide was being broken down into the dipeptide Gly-Phe and the free carboxy-terminal amino acid, Methionine. After a total of 200min-digestion the Met-Enkephalin peak on the HPLC-traces had disappeared completely. The rate of digestion of Met-Enkephalin by the immobilized DAPI was approximately 20% the rate of digestion using soluble DAPI under identical conditions. The fact that the digestion was slower and therefore more controllable provides a useful technique for the digestion of peptides and proteins for sequential analysis. In particular, it may be of use in conjunction with the HPLC-continuous flow FAB-mass spectrometry techniques for, among other things, sequential analysis. (see Chapter 1)

3.4.7 Cyanogen Bromide Activated Sepharose Immobilization

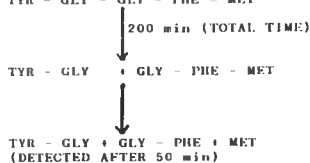
Immobilization of DAPI by hydrophobic bonding to commercially available octyl- or phenyl-Sepharoses was far superior to immobilization on cyanogen bromide activated Sepharose (see Fig. 3.4.7). DAPI immobilized by the latter method was inactive. This could be due to two possible reasons. Firstly, the pH at which immobilization is required to take place may be too high (too alkaline) for DAPI to remain stable for any

A

DIGESTION OF METHIONINE - ENKEPHALIN

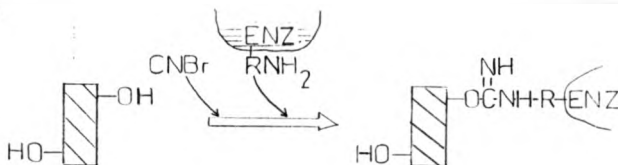
ENZYME IMMOBILISED ON OCTYL - SEPHAROSE

SUBSTRATE: TYR - GLY - GLY - PHE - MET



DETECTION BY REVERSE PHASE HPLC AT 214 nm

B



A Fig. 3.4.6 Digestion of Met-Enkephalin

B Fig. 3.4.7 Immobilization onto Cyanogen-Bromide-Activated Sepharose

length of time. However, reduction of the pH of the immobilization reaction to pH7.5 did not lead to the production of active immobilized DAPI. Alternatively, the covalent bonding required may have deleterious effects upon the secondary, tertiary and even quaternary structures of this multi-subunit enzyme, which could have caused activity loss in the preparation. This phenomenon has been recorded before for some multi-subunit enzymes. (Ref. 61).

CHAPTER 4

Purification and Characterization of AcAP

Section 4.1 Introduction

Along with DAPI, this enzyme will be used in sequence studies. As for DAPI this enzyme has been purified by a modified procedure to include TSK-HPLC. The methods used for the characterization have added evidence to the fact that the enzyme one isolated was in fact AcAP; in an apparently homogeneous form. Once again, the most important properties to be obtained for accurate characterization, namely molecular weight and isoelectric focal point have been determined and are both close to literature sources.

Inhibition studies have also been carried-out, but the results are not as clear-cut as the above factors - and although some evidence can be attributable to a preparation of AcAP - others cannot so easily. This forms the basis of an interesting discussion.

Together with Chapter 5, detailing substrate specificity studies the evidence for the enzyme preparation obtained in this study being AcAP is highly favourable. It is with confidence that one can say that the enzyme sample isolated by the given method is AcAP in a form suitable for substrate/sequence studies.

4.2 Materials

4.2.1 Purification of Acetylaminopeptidase (AcAP)

Porcine liver was obtained 4h after slaughtering. DE52 cellulose from Whatman laboratories. Sephacryl S-300 from Pharmacia-LKB, TSK-HPLC column from Amicon Ltd Glos UK (7.5x300mm) used on a Waters (G3000SW) Associates Model 6000A pump, a U6K injector and 441UV detector at 280nm.

4.2.2 AcAP Assays

N-AcetylAlanyl-p-nitroanilide(AANA) purchased from Sigma Chemical Co. Ltd., Poole, Dorset, UK.

4.2.3 Characterization of AcAP

4.2.3.1 Molecular Weight Determination

- (a) HPLC Method TSK apparatus as for Sect.4.2.1. Calibration proteins from BDH Ltd.
- (b) PAGE Estimation Calibration using Bovine Serum Albumin (BSA) Cohn Fraction V, 96-99% pure and essentially salt free from Sigma Chemical Co. Poole.

4.2.3.2 Isoelectric Point Determination

Performed ampholine gel (pI3-10) on a LKB flat-bed electrophoresis unit. pI standard proteins (pI4.7-10.6) from BDH chemicals.

4.2.3.3 Activity Stain

AANA as for Sect.4.2.2. Polyacrylamide gel components from BDH chemicals.

4.2.3.4 Peptidase Contamination

Chymotrypsinogen A, trypsin and pepsin from Sigma Chemical Co, Poole.

4.2.3.5 Inhibition Studies

Salts of the metals, Zinc and Copper obtained from Sigma Chemical Co, Poole.

4.2.3.6 Storage Studies

AANA from Sigma Chemicals Ltd, Poole, Dorset.

4.3 Experimental

4.3.1 Purification of AcAP

Method of Tsunasawa and Narita, Methods in Enzymology p552-61, (Ref. 42).

4.3.1.1 Extraction

Porcine liver (500g) was defatted, thinly chopped and suspended in two volumes of sodium phosphate buffer (5mM, pH7.2) containing 2-mercaptoethanol (10mM) and EDTA (1mM) (MEP-buffer)-total volume, 800mls. The suspension was thoroughly homogenised in a glass-walled Waring rotary blender for 10mins. The homogenate was centrifuged at 10,000g (7,750 rpm, 6"rotor, MSE-18) for 10mins and the supernatant collected.

4.3.1.2 Ammonium Sulphate Fractionation

The supernatant obtained was subjected to a commonly utilized method of protein purification. An ammonium sulphate "cut" or fractionation is performed between saturation levels of 20% and 50%. Ammonium sulphate (Analytical Reagent grade) (107.16g in 940mls of supernatant) was stirred into the solution and left overnight (20% saturation) at 4°C. The precipitate was centrifuged off at 10,000g for 30mins as before. The resultant supernatant was then made up to 50% saturation by the addition of further ammonium sulphate (further 176g in 930mls of supernatant). This solution was left stirring at 18°C for 5hrs. The precipitate was again obtained by centrifugation (10,000g for 30 mins) and resuspended in cold 0.2M NaCl-MEP buffer (100mls) and the solution dialyzed overnight

against several changes of the same buffer. Visking tubing with a molecular weight cut off of 10000 daltons was used for dialysis, prepared for use by first boiling in a solution of sodium bicarbonate @250mg/100mls of distilled water with a trace of EDTA and then rinsed in a boiling water bath.

4.3.1.3 Heat Treatment

The dialyzed solution was first filtered using a UM-10 filter (Molecular weight cut-off 10,000) on a Diaflo ultrafiltration unit (65ml volume) at 65 psi. The volume was reduced to 250mls. The concentrated enzyme solution was then subjected to heat treatment. This was achieved by heating the solution, with continuous stirring to 60°C in a conical flask by the use of a water bath. After 5 minutes the solution was chilled rapidly in ice. Precipitated materials were then removed by centrifugation at 10,000g for 30mins.

4.3.1.4 DEAE-Cellulose Chromatography

The supernatant was applied to a DE52-cellulose column (68cmx2.5cm diam.) equilibrated with 0.2M NaCl-MEP buffer (pH7.2).

Equilibration buffer (1.25l) was washed through the column after sample application (125x10ml fractions) prior to the application of a salt gradient (0.2-0.6M NaCl-MEP buffer pH7.2, 2 l volume). Any residual protein was eluted by the application of a 2.0M NaCl-MEP buffer (pH7.2) solution.

4.3.1.5 Gel Filtration Step

Further separation of the enzyme was carried out using Sephacryl S-300 superfine resin (Molecular Weight Separation range, 2×10^3 - 4×10^5 daltons), swollen and equilibrated with 0.2M NaCl-MEP buffer (pH7.2) and packed into a column (75cmx5cm diam.). The absorbance (A_{280nm}) of the effluent was monitored (as for DE52 chromatography, sect. 4.3.1.4) to give an indication of protein elution across the profile.

The pooled fractions which showed acetylaminopeptidase activity from the DE52-chromatography step were ultrafiltered (as in sect. 4.3.1.3) down to 10mls and applied to the column. Elution was achieved using the equilibration buffer (10ml fractions) at room temperature over 15hrs.

4.3.1.6 TSK-HPLC Purification

A TSK-High Performance Liquid Chromatography column was equilibrated with 5mM Phosphate buffer (pH 7.2) including 2-mercaptoethanol (10mM) on a Waters associates system (see Sect. 4.2.1). Enzyme containing fractions from the gel filtration step (Sect. 4.3.1.5) were again pooled, ultrafiltered and aliquots (100 μ l) were injected onto the column. Detection was by flow monitoring at 280nm, flow rate was set at 0.4mls/min and detection limit at 0.2 absorbance units full scale (a.u.f.s). The profile obtained (see Results Sect. 4.4.1.6) indicated the fractions collected (0.4mls) for assay. Identification of the relevant enzyme containing fractions enabled repeated HPLC purification steps to be carried out. All fractions from these repeated steps were pooled and ultrafiltered on Centricon-10 units (AMICON LTD). The enzyme solution was stored at 4°C until required.

4.3.2 Acetyl Aminopeptidase Assay

4.3.2.1 Specific Activity Assays

The assay for AcAP relies on its acetyl amino acid cleaving function by the monitoring of the cleavage of the substrate, N-Acetyl-L-Alanyl-p-Nitroanilide (AANA). Cleavage occurs at the amide bond between the alanine and p-nitroanilide groups (see fig.442b). The resultant products are thus Acetyl-alanine and p-nitroaniline. The latter is monitored spectrophotometrically at 405nm (yellow). Specific activity estimations were obtained from the assay above and protein concentration assays (Ref 59). Actual activities were estimated by direct comparisons with a standard p-nitroaniline curve (fig.442c).

A stock solution of AANA (4mM) was made up in Sørensen's buffer (0.1M, pH8.3) including sodium chloride (0.2M). The assay was carried out using 500 μ l of this stock solution which was incubated at 37°C in the jacketed cuvette holders of a SP1800 double-beam spectrophotometer. The assay was initiated by the addition of a known volume of enzyme solution and monitoring was carried out at 405nm at 37°C. Activities were calculated from initial rates of the reaction.

4.3.2.2 Rapid Assay

This assay method was developed for a quick qualitative response and uses the same principles as above (Sect. 4.3.2.1).

An AANA stock solution (4mM) in 0.2M NaCl-MEP(pH7.2) was prepared and

aliquots (100 μ l) were placed in separate wells of a micro-ELISA plate. Enzyme samples (column fractions) (50-100 μ l) were added to the wells. The formation of p-nitroaniline was monitored using a micro-ELISA recording spectrophotometer (with a 410nm filter) after 10 minutes incubation at the required temperature.

Blank readings obtained from assays were

- (a) No enzyme was added (50 μ l buffer + 100 μ l substrate)
- (b) No substrate was added (only 100 μ l buffer + enzyme), to determine latent or spontaneous substrate hydrolysis under such conditions.

The K_m - and V_{max} - values of the assay are calculated, and details of which are outlined in Chapter 5 (Sect. 5.3.1).

4.3.3 Characterization of AcAP

4.3.3.1 Molecular Weight Determination

4.3.3.1 (a) TSK-HPLC Method

Molecular weight determination was carried out using TSK-column chromatography and electrophoresis (Sect. 4.3.3.1(b)) and molecular weight standards.

The TSK-Column on a Waters HPLC system (see Section 4.2) was equilibrated with Sérensons buffer (Ref. 58) (50mM, pH7.2) and run at a flow rate of 1ml/min, pressure of 425 psi and detection at 280nm. Molecular

weight markers (Δ 1mg of each) were solubilized in the equilibration buffer, and aliquots (10 μ l) were injected onto the HPLC apparatus and the elution times recorded. The markers with their molecular weights, used were:- Ferritin (458kd), Catalase (240kd), Aldolase (158kd), Bovine Serum Albumin (68kd), Hen Egg Albumin (45kd), Chymotrypsinogen A (25kd) and Cytochrome C (12.5kd). A standard curve of elution time vs molecular weight was constructed. A reference compound, Blue Dextran (Approx. Weight = 2×10^6 d) was also chromatographed to obtain the void volume elution time.

A sample of purified AcAP (50 μ l, 0.1mg Protein) was injected under identical conditions and the elution time recorded. From the calibration curve constructed an estimate of the molecular weight for AcAP can be determined.

4.3.3.1 (b) Polyacrylamide Gel Electrophoresis Estimation of the Molecular Weight of AcAP using Glutaraldehyde formed Polymers of Albumin (BSA).

(From Payne, BioChem.J.(1973) Vol.135 Ref. 64)

Glutaraldehyde is well documented for its ability to react with proteins and to produce cross linked aggregates. A procedure devised by Payne has yielded covalently-linked soluble protein oligomers. It is applicable to a wide range of proteins, for soluble polymers between 3×10^4 - 2×10^7 daltons. It is a very useful procedure for use in molecular weight determinations. The inherent similarities between the formed oligomers make them superior to commercially available molecular weight marker kits, which can have marked differences between the proteins used.

Bovine Serum Albumin (Fraction V, 96-99% pure from Sigma Chemical Co. Ltd.) was dissolved in TRIS/Glycine buffer (200mg/ml, 0.25M TRIS, 1.92M Glycine) containing 1% sodium dodecyl sulphate (SDS), (pH8.3). To samples of the protein solution (150, 100, 50, and 12.5 mg/ml, total volume of each 200 μ l) was added a glutaraldehyde solution (5% v/v) with simultaneous stirring (6, 4, 2 and 0.5 μ l respectively). Mixing was continued for 1min. and the solutions incubated at 18°C for 24 hrs.

An AcAP freeze dried sample was dissolved in the TRIS/Glycine buffer (10mg/ml by Hartree protein assay) containing 2-mercaptoethanol (0.5% v/v). All samples were mixed with sucrose (50% v/v) and gel buffer (see below, 5 μ l) after boiling on a water bath for 30min., centrifugation to remove insoluble matter, and dilution to protein concentrations ready for electrophoresis (10mg/ml).

A 10%-polyacrylamide-SDS gel (18cm x 16cm) was used for the resolution of all samples. Electrophoresis was carried out at 100V constant voltage (17mA) for 30min and then 200V constant voltage (35mA) for a further 120min. The resolved gel was stained in a Coomassie-blue R250 (0.1%) solution in water:methanol:glacial acetic acid (5:5:2) for 16hrs and destained in the same solution, minus the Coomassie blue dye, for 24hrs.

Table 4.3.3.1 (b) SDS-PAGE Composition

Gel Composition	Stacking Gel	Resolving Gel	Reservoir Buffer
ACRYLAMIDE:BISACRYLAMIDE (30:08)	2.5ml	10ml	-
STACKING GEL BUFFER (A)	5.0ml	-	-
RESOLVING GEL BUFFER (B)	-	3.75ml	-
RESERVOIR STOCK BUFFER (C)	-	-	100ml
10% SDS	0.2ml	0.3ml	-
1.5% AMMONIUM PERSULPHATE (APS)	1.0ml	1.5ml	-
DISTILLED WATER	11.3ml	14.45ml	900ml
TEMED	15µl	15µl	-
TOTAL VOLUME	15ml	30ml	1000ml

Buffer A - 0.5M TRIS/HCl (pH6.8; 6g TRIS in 40ml distilled water titrated with 1.0M HCl and made up to 100ml).

Buffer B - 3.0M TRIS/HCl (pH8.8; 36.3g TRIS titrated with 48ml 1.0M HCl and made up to 100ml).

Buffer C - 0.25M TRIS/ (pH8.3, 30.3g TRIS, 144g Glycine 10g SDS 1.92M Glycine/1% SDS dissolved and made up to 1000ml).

All buffers were filtered through a Whatman No. 1 paper before use. Storage was at 4°C.

The distance moved from the Anode (well bottoms) was measured and plotted against the oligomer molecular weights (6.7kdxn; n=1-). The distance moved by the dissociated AcAP monomers was measured and their molecular weights determined from the calibration curve. Molecular weights of undissociated AcAP-associated bands can also be determined using this technique. Thus, this method can give an indication of the subunit composition as well as molecular weights of the AcAP enzyme.

4.3.3.2 Isoelectric Focussing Point (IEP) Determination

The IEP determination was carried out using precast gels; isoelectric point markers and Coomassie blue R250 staining.

The IEP is a key characteristic (with molecular weight) and represents the pH at which the net charges of the various amino acids of the enzyme is zero or neutral.

An LKB-ampholine PAG precast plate (pI3.5-9.5) (Product No. 1804-101) containing Ampholine carrier ampholytes designed to set up an isoelectric gradient between the poles at opposite ends of the gel when an electric current is applied, is cut to the required length and placed on the cooling plate (10°C) in a Multiphor I flat-bed electrophoresis unit from LKB sandwiched between the plate and gel are the locating template and thin paraffin oil layers for insulation purposes.

An isoelectric point marker kit was purchased from BDH chemicals (Poole, UK) which contained markers for the pI range 4.7-10.6. These proteins were;

Isoelectric Point Marker Kit Components

PROTEIN	pI	MOLECULAR WEIGHT
C-PHYCOCYANIN (Anidulans)	4.75/4.85	232kd
AZURIN	5.65	14kd
TRIFLUOROACETYLATED MYOGLOBIN MET (PORCINE)	5.92	17kd
MYOGLOBIN MET (PORCINE)	6.45	17.6kd
MYOGLOBIN MET (EQUINE)	7.30	17.6kd
MYOGLOBIN MET (SPERM WHALE)	8.30	17.8kd
CYTOCHROME C (HORSE HEART)	10.6	12.4kd

This particular kit was chosen because of:

- (1) Its applicable range.
- (2) Coloured components - making it simple to follow the extent of focussing during the run and,
- (3) Many of the components have similar molecular weights which would help to offset any effects that the molecular weight of a sample may have upon the migration and focussing under the set conditions samples of AcAP (purified on TSK-HPLC apparatus and concentrated on Centricon-10 mini-ultrafiltration devices purchased from Amicon Ltd) (10µl, 5mg/ml) were applied to small sample application strips [Filter paper (3mmx8mm)] placed on the gel surface (3cm from Cathode) in positions 2,4,6,8 and 10. In a similar way, samples of the marker- kit protein solution (5µl of 32mg/ml solution)

were applied to application strips in positions 1,3,5,7 and 9. Electrode strips (Filter paper, 5mm wide) were prepared, the anodic strip was soaked with phosphoric acid (1M) and cathodic strip soaked in sodium hydroxide (1M). The electrodes were connected to the electrode strips across the gel and focussing was carried out at 1200 volts constant voltage and up to 9mA current (10 watts maximum power) regulated on a Pharmacia power-pack. Focussing was carried-out for 2hrs, with the sample application pieces removed half way through as recommended by the manufacturers. The gel was removed and 'fixed' using trichloroacetic acid (57.5g/500ml ddH₂O) and sulphosalicylic acid ((17.25g/500ml distilled water) which removed the ampholines and precipitated the proteins, for one hour. Prior to staining, the gel was washed with destaining solution; ethanol:acetic acid:distilled water (5:1:6:13.4). Staining was carried out in the destaining solution containing Coomassie Brilliant Blue R250 (0.1%, filtered and heated to 60°C prior to use) for 10mins. Destaining was achieved with several changes of the aforementioned solution. Preservation of the gel was achieved using glycerol in destaining solution (1 part to 10 parts) for 1 hour. The gel was then allowed to dry to stickiness, and then covered with a thin plastic film.

The IEP calibration curve was constructed by measuring the distance of the bands from the cathode and attributing these to the isoelectric kit marker proteins and their respective isoelectric points. The bands used appeared as blue stained strips. This method of calibration curve construction has inherent advantages over the other two most commonly used methods. Use of a soft-surface electrode does not allow good equilibration with the gel surface, and the second method of elution of

the ampholines into a small volume of distilled water (small gel strips cut at 90° to the applied electric field) and then measuring the pH of the solution, is both tedious and gives rise to dilution and temperature inconsistencies and problems.

The isoelectric point of AcAP was hence determined by direct reading from the calibration curve.

4.3.3.3 Activity Stain of AcAP on a Polyacrylamide Electrophoretic Gel

A non-dissociating 7.5% resolving gel was used for this analysis with a 5% loading gel overlayed. The actual components of the gel are listed in section 4.3.3.1 with the exception of the use of 7.5ml of Acrylamide:Bisacrylamide (30:0.8) solution and omission of the SDS. The volumes lost were made-up with distilled water. Similarly, for the stacking gel, water replaces the SDS component.

The object of this analysis was to identify AcAP in an electrophoretically resolved polyacrylamide gel and distinguish it from other proteins present in an AcAP solution. (Solution was that used for final purification of AcAP on TSK-HPLC, section 4.4.1). The actual activity stain was achieved using the commonly used substrate of AcAP, namely Acetyl-alanyl p-nitroanilide.

Samples of AcAP solution (0.11mg/ml) were applied to the gel (50 µl to positions 2, 3, 9 and 10, 25 µl to positions 4 and 8 and 10 µl to positions 5 and 7. Positions 1, 6 and 11 were left blank). Bromophenol blue

(0.002% in 10% glycerol) was added to each sample prior to loading to provide a means of monitoring the extent of electrophoresis as it co-runs with the gel boundary. Addition also aids the loading of samples. The gel was developed by running at 200V constant voltage (23-25mA current) at 18°C for 2 hours using the reservoir buffer, TRIS-Glycine (pH8.3) (see section 4.3.3.3(b)). After electrophoresis the gel was split down the centre (along track 6). One half was stained with Coomassie Brilliant Blue (0.1%) in H₂O: methanol:acetic acid (5:5:2) for 4 hours, and destained in methanol (30%), acetic acid (10%) and distilled water (60%). The second identically-loaded half was subjected to the following activity stain. Acetylalanylnitroanilide (AANA, 4mM in 0.2M NaCl-Sørensen's buffer, pH8.3) was smeared over the gel thinly. The formation of the product of AANA hydrolysis by AcAP, namely p-nitroaniline was monitored by a micro-ELISA monitor at 410nm as used in the rapid assay of AcAP (section 4.3.2.2). Localized formation was observed which indicated the position of the enzyme (active) in the gel. The position was then compared with the Coomassie protein stained half, and hence the position of the enzyme band was ascertained. Thus, this result may form the principle behind a purification step of AcAP into a highly purified form for sequence or characterization studies.

Section 4.3.3.4 Peptidase Contamination

Any peptidase-type contaminant enzymes in the AcAP preparation may be responsible for some activities and characteristics were incorrectly attributed to AcAP. Therefore, it is desirable to eliminate this possibility so that a step towards the correct characterization of AcAP can be made.

By direct comparison of an AcAP preparation and the purchased, common peptidases, Chymotrypsinogen A, trypsin, and pepsin by TSK-column it is believed that this possibility can be deleted or deemed worthy of further analysis.

Samples of each enzyme were analyzed by TSK-column HPLC elution with detection at 280nm with 50mM phosphate buffer + 2-mercaptoethanol (1mM)(pH7). Aliquots (10 μ l, 0.1mg/ml) of each enzyme in the elution buffer were injected onto the column and the elution profiles compared. TSK-column HPLC is based upon gel filtration methodology and hence the molecular weights of all components of the sample will form the major principles behind their elution profiles. Comparison of a purified AcAP samples profile could thus be made and the presence of contaminant proteases in one's samples verified or ruled-out.

4.3.3.5 Inhibition Studies

Aliquots of AcAP (500 μ l, 1mg/ml) were incubated with separate solutions of metallic cations (1mM) in MEP-buffer and the activities of the enzyme-metal ion solution/complex was assayed by removal of portions for AANA assay (section 4.2.2). The changes in activity were monitored by assay from time t=0 to t=2hrs after incubation at 18°/37°C. Controls included monitoring the effects of the metal-ion solution upon AANA spontaneous hydrolysis and all assays were compared to that rate of hydrolysis achieved by the use of native enzyme. The results were analyzed by plotting the percentage retained activity against time.

4.3.3.6 Storage

The activity of aliquots of AcAP stored at 18°C, 4°C and -20°C in MEP-buffer was assayed after equilibration back to 37°C by the AANA assay. This study was designed in an attempt to formulate ideal storage conditions for maximal enzyme activity retention.

4.4 Results and Discussion

4.4.1 Purification of AcAP

The purification of AcAP was monitored by determining the enzymic activity and protein concentration (Hartree Assay, Ref. 59) of aliquots taken during the various stages employed. The results of these assays are tabulated in Table 4.4.1.

The final enzyme preparation was shown to be homogenous by electrophoretic study (section 4.4.3.1 (b)) and TSK-HPLC (section 4.4.3.1 (a)). The preparation was also shown to be free from the common peptidase enzymes i.e. trypsin, pepsin, and chymotrypsinogen A by analysis of their chromatographic mobilities when compared under identical conditions to the mobility profile of an AcAP sample. Standard methods used to identify the presence of peptidases in a preparation include substrate specificity studies and enzyme inhibition by specific inhibitors such as the Soybean Trysin/Chymotrypsinogen inhibitor (Bowman-Birk Inhibitor, Ref: Birk, Y.Int. J.Peptide Protein Res. 25, 113 (1985)).

This preparation was deemed suitable for accurate characterization by the methods outlined in this study - Molecular weights, isoelectric point and metal inhibition studies were all carried out, and the results are shown and discussed in the appropriate section.

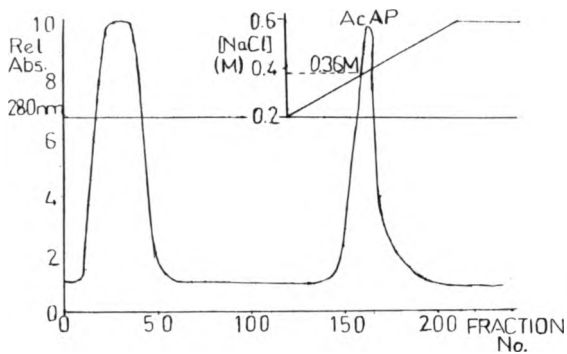


Fig. 4.3.1.4 AcAP Elution following DEAE-Chromatography

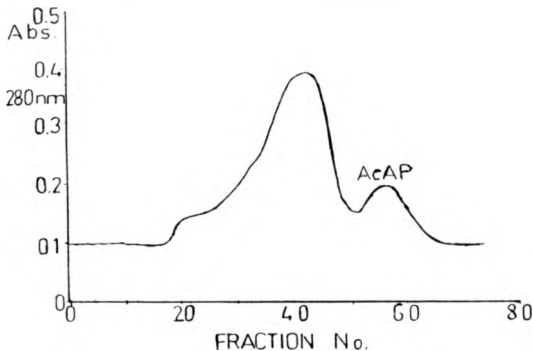


Fig. 4.3.1.5 AcAP Elution following Sephacryl S-300 Gel Filtration Chromatography

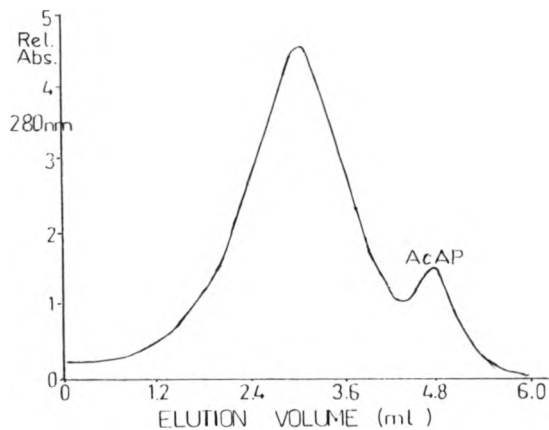


Fig. 4.3.1.6 AcAP Elution following TSK-HPLC Chromatography

Table 4.4.1. Purification of Acetylaminopeptidase (AACP)

Stage	Category	Volume (mls)	Protein Concentration (mg/ml)	Total Units (U)	Specific Activity (U/mg P)	Purification Factor (X)
Extraction		1,100	10.5	4,650	0.4	1
	Ammonium Sulphate Fractionation (60% Pellet Resuspended)	250	6.03	2,700	1.79	4.5
	Post DEAE Step	200	1.40	1,050	3.75	9.4
	Post S300 Step	10	2.25	450	20.0	50
	Post TSK HPLC Step	2.0	1.0	340	170.0	425

4.4.2 AcAP assays See Figs. 4.4.2 (a), (b) and (c).

4.4.2.1 Specific Activity Calculations

The specific activities of AcAP at various stages throughout purification are detailed in Table 4.4.1. The determination of these values are simple to obtain by using a rapid colorimetric assay which enables results to be obtained in a few minutes. This is in contrast to the method used by Tsunawasa et al (1975, Ref.41) which is a variant of the ninhydrin method of Yemm and Cocking (1955, Ref. 51). Not only does this assay use toxic reagents but it also takes up to 1½ hours, utilizing several steps to use. The method one uses is also preferable to that utilizing a fluorimetric step. Fluorescamine (FL, 4-phenylspiro-(furan-2(3H),-1' phthalan -3,3'-dione) introduced by Weigle et al (1972) and analyzed by Chen et al (1978, Ref. 69) with respect to its reaction with amino acids, is one such reagent to use. However, it is not simple to use accurately. Furthermore, it has little understood reaction mechanisms, i. e. it exhibits fluorescent changes due to the inclusion of some solvents. Therefore, the colorimetric assay by p-nitroaniline production monitoring, has been extensively used in this study because of its ease of use and adaptability. (See also Rapid Assay, section 4.4.2.2 and Activity Stain, section 4.4.3.3).

4.4.2.2 Rapid Assay

The adaptability of the AcAlapNA assay is underlined by the rapid assay of AcAP possible using this substrate. This enabled the rapid assay of the column fractions in the purification procedure. Thus, the position

of the eluted enzyme in the protein profile was obtained within 60 minutes with as much accuracy as previously reported assays in a fraction of the time. This rapid assay was not designed for quantitative analysis but rather, for qualitative analysis. The actual enzyme content obtained was determined by the assay outlined in section 4.3.2.1 once the position of the enzyme was ascertained, thus saving money, time and enzyme material.

The pH-profile, maximal velocity and equilibrium constants for this assay were obtained and are given in Chapter 5, sections 5.4.1 and 5.4.2.

4.4.3 Characterization of AcAP

4.4.3.1 Molecular Weight Determination of AcAP

4.4.3.1 (a) TSK-HPLC Method

The elution times of the standard molecular weight markers and reference compounds as well as a sample of AcAP previously purified on TSK-HPLC equipment (section 4.2.1) under the stated conditions are shown in table 4.4.3.1 (a).

Table 4.4.3.1 (a) Elution Times of Standard Protein Samples

(used to construct a standard curve for the molecular weight determination of AcAP).

Sample	Mol. Wt (Kdal)	Log ₁₀ Mol. Wt	Elution Time (min)	Corrected* Elution Time (min)
Blue Dextran	2×10^3	-	5.8	-
Ferritin	450	2.65	7.0	1.2
Catalase	240	2.38	8.9	3.1
Aldolase	158	2.30	11.9	6.1
BSA	68	1.83	13.0	7.2
HEA	45	1.65	15.2	9.4
Chymotrypsinogen A	25	1.39	21.35	15.55
Cytochrome C	12.5	1.09	24.95	19.15

(*Minus elution time of Blue Dextran)

A direct plot of the corrected elution times versus molecular weight (\log_{10}) of the components was constructed. The elution time of a purified sample of AcAP was determined under identical conditions and its molecular weight determined from the standard curve. The estimated molecular weights of AcAP was found to be 295 ± 20 K daltons. This value was in close agreement with reported values and it is further confirmed by the second method of molecular weight determination of AcAP by PAGE (Section 4.4.3.1. (b)).

CORRECTED
ELUTION TIME (min)

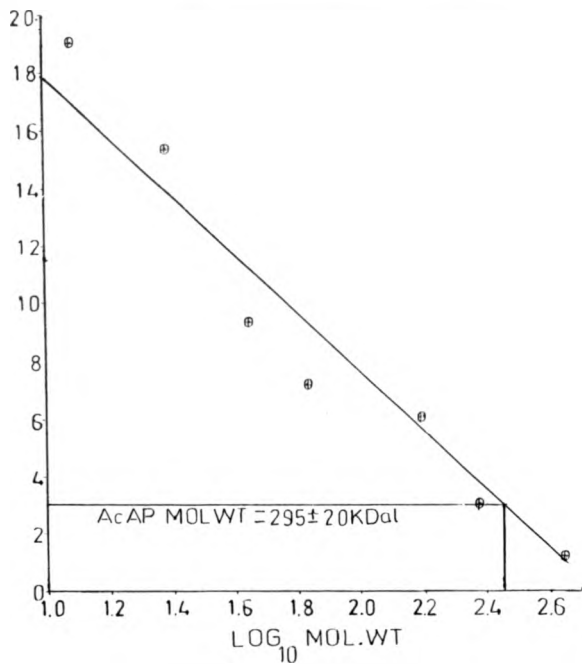


Fig. 4.4.3.1 (a) TSK-HPLC Standard Curve for the Determination of the Molecular Weight of AcAP

4.4.3.1 (b) Glutaraldehyde Linked BSA Method for the Molecular Weight Estimation of AcAP

The results obtained (Fig. 4.4.3.1 (b)) show a linear relationship between the molecular weights (\log_{10}) of the formed - BSA oligomers and their mobility down the gel. The monomeric band was identified by running a sample of native BSA (67 kdal) alongside on the same gel. From this value the other bands detected were assigned molecular weights as multiples of 67 kdaltons as one goes up the gel. This represented the bands due to the dimer, trimer, tetramer etc. A standard curve was constructed from these results. Bands up to and including the heptamer of BSA at 469 kdaltons were possible to identify, other bands above this were noticeable, but accurate assignment was not possible.

From the standard curve a value for the molecular weight of AcAP was obtained and was found to be 300 ± 30 kdaltons, a value very close to that obtained by TSK-HPLC.

The subunit structure, and their molecular weights of AcAP was not possible to determine in this study. This may be attributable to incomplete dissociation of the enzyme under the conditions used. A change in the conditions and the gel composition used would give a clearer insight into the subunit composition, but, of course such findings would have to be corroborated using other techniques. Such investigations were not undertaken in this study.

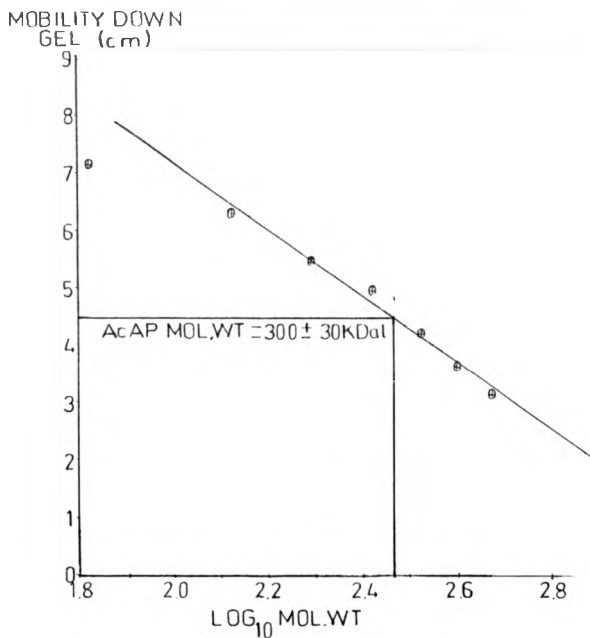


Fig. 4.4.3.1 (b) Glutaraldehyde-Linked BSA-PAGE Standard Curve for the Determination of the Molecular Weight of AcAP

4.4.3.2 Isoelectric Focussing Point Determination

The results obtained (Fig. 4.4.3.2) show a good linear relationship between the isoelectric point marker components and their mobility across the preformed isoelectric gradient.

The distance moved by a purified sample of AcAP (6.9 cm from the Cathode) indicates by reference to the standard curve, an isoelectric point of 5.14. This value is in quite good agreement to those values obtained by other workers (See Introduction, Chapter 1) with this enzyme, and serves to reinforce the evidence obtained for the correct identification of AcAP. This method of isoelectric point determination is highly reproducible and rapid, and shows marked improvements over the method used in Chapter 2 for the isoelectric point determination of DAPI.

However, it should be noted that this method is more expensive than that employed in Chapter 2.

4.4.3.3 ActivityStain

The results obtained (Fig. 4.4.3.3) indicate that:-

- (a) the enzyme is still active under the electrophoretic conditions used, and
- (b) the simple addition of Ac-L-Ala-pNA (AcAP substrate) is capable of identifying the band due to AcAP as being that of the central band of three indicated by Coomassie Blue staining. Limitations of this method include non-identification of co-electrophoresed sample components (i.e. two different components at the same band position)

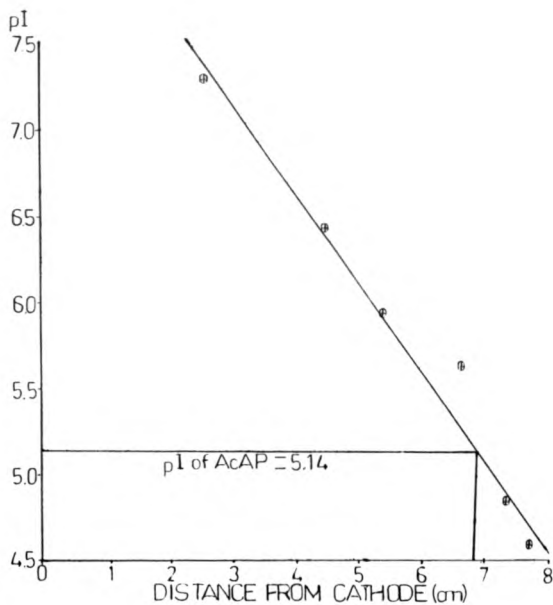


Fig. 4.4.3.2 Calibration Curve for the Isoelectric Point Determination of AcAP

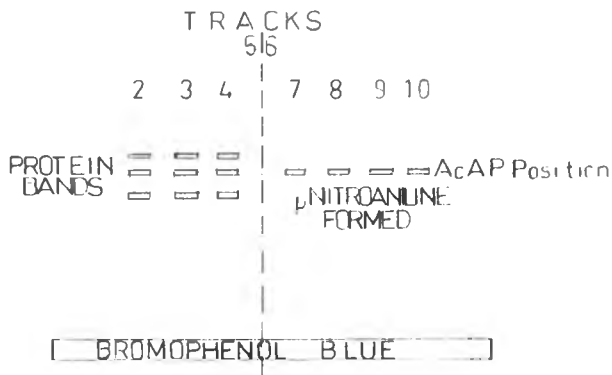


Fig. A.4.3.3 p-Nitroaniline Activity Stain of AcAP on a Non-Dissociating PAGE System

or run very close to one another. To overcome the latter problem one could excise the band from the gel by scalpel and stain by placing the gel strip directly into a solution of substrate. Formation of pnitroaniline on the gel surface was quite rapid, but not as fast as that observed using the same amount of enzyme which had not been electrophoresed. The enzyme, after electrophoresis, may therefore have lost some activity but the difference is much more likely due to steric effects. The substrate is only applied to the gel's surface, and, if adsorption into the gel is not observed then obviously, enzyme which is present at a location throughout the width of the gel would not come into contact with the substrate.

However, this simple assay could be used to identify AcAP in complex AcAP mixtures and may form the basis of a new step for the ultra-purification of AcAP by preparative PAGE.

Furthermore, with adaptations, the identification of other proteases and peptidases present in a mixture could be ascertained - but further work needs to be applied to this hypothesis.

4.4.3.4 Peptidase Contamination

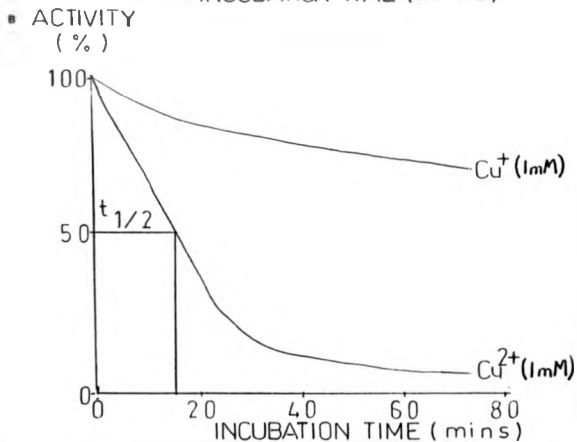
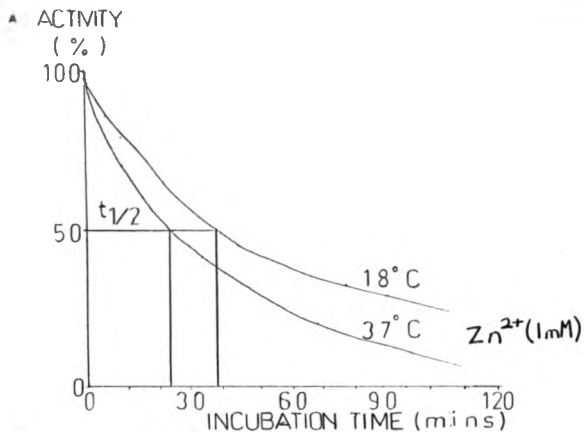
The elution profile of AcAP was very similar to that obtained in section 4.4.3.1 (a), used for the molecular weight estimation of AcAP. Elution profiles of Chymotrypsinogen A (23Kdal, Ref 3), Trypsin (23Kdal, Ref 40) and pepsin (36Kdal, Ref 3) were significantly different. These protease enzymes were eluted much earlier under the defined conditions and hence, by this method, one could confidently state that any protease/peptidase

activity exhibited by AcAP was probably not due to the presence of any of the above enzymes. This method excludes any need to refer to more ambiguous methods of identifying protease contamination such as substrate specificity and heavy metal inhibition because these parameters for AcAP are not fully defined as yet and may overlap with the proteases - hence, a false result may occur.

4.4.3.5 Inhibition Studies

The results obtained are shown in Figs 4.4.3.5 (a) and (b). Zinc inactivation of AcAP is evident from the plots and this inactivation is shown to accelerate as the temperature is raised to 37°C. This is understandable if one considers that if temperature is raised the reaction rate indicated by an enzyme turnover rate is faster and hence inactivation becomes faster; Taunasawa et al (Ref. 41) state that zinc does not appear to inhibit the enzyme. This disagreement in the results could be attributable to:-

- (a) The enzymes studied are not exactly the same, this possibility could also be backed-up by the discrepancies in molecular weight estimations and isoelectric point calculations shown in this study. However, the two molecular weight values quoted in the reference differ greatly and therefore, not too much emphasis should be placed on this.
- (b) Taunasawa's inhibition studies only covered incubation for 10 minutes prior to assay. My study involved the monitoring of enzymic activity at various times between t=0 and t=80 minutes and;
- (c) The substrates used are not the same i.e. Taunasawa favoured the



▲ Fig. 4.4.3.5 (a) Zn^{2+} Inactivation of AcAP using AANA as substrate

■ Fig. 4.4.3.5 (b) Cu^{2+}/Cu^{+} Inactivation of AcAP using AANA as substrate

ninhydrin detection of hydrolyzed products from Ac-L-Met-L-Thr at pH7.2. However this study utilized the detection of p-nitroaniline following hydrolysis of Ac-L-Ala-pNA at pH8.3.

Without further study, this discrepancy remains to be clarified.

Copper inhibition of AcAP in this study, agrees with those studies carried out by Tsunasawa *et al.* Cu^{2+} inhibition is quite rapid in this study, with only 60% of the original activity remaining after 10 minutes; and only 5% after 70 minutes. Again, lack of total inhibition after 10 minutes could be attributable to the different assay and conditions employed.

It is worth noting that there appeared to be some inhibition with Cu^+ , although by no means as obvious as with Cu^{2+} . This result is included for discussion, the slow inhibition may yet prove to be artefactual due to other heavy metal contamination, or may be genuine, and thus, may cast more light upon AcAP's mode of operation.

4.4.3.6 Storage

The formulation of ideal storage conditions for an enzyme, have to take into account several factors. Temperature, pH, buffer components in solution or lyophilized powders are the most important. This study attempts to formulate an ideal storage temperature for the enzyme, by analyzing the percentage activity remaining of AcAP samples, after storage at three different temperatures after 12 hours.

After storage at -20°C the enzyme sample showed less than 5% remaining activity after assay. This result is in close agreement to that observed by Taunasaawa and Narita (Ref. 42). The reason postulated, was a loss of activity due to the presence of 2-mercaptoethanol, which was also present in my samples buffer.

Storage at 4°C caused less than 10% loss in activity, a figure close to Taunasaawa's after storage at 4°C for 1 month. This indicates that the majority of inactivation occurs within the first hours of incubation.

A retained activity of 80% was observed after storage at 18°C . This is consistent with a common observation of enzyme sample in that, the processes of activity loss become accelerated as the incubation temperature is raised.

From this simple study it was concluded that for the purified AcAP's storage, a temperature of 4°C was chosen - in the presence of 2-mercaptoethanol.

Chapter 5

Analysis of some possible substrates for Acetyl Amino-peptidase (AcAP)

5.1 Introduction

This chapter only touches upon a wide ranging topic, the substrate specificity of an enzyme. It is incorporated here in an attempt to indicate the 'directions' and extent to which AcAP can hydrolyze substrates and as a comparison with published material which may provide more evidence for the correct identification of the purified AcAP utilized in this study, in addition to Chapter 4 (this study).

5.2 Materials AcAP purified as detailed in Chapter 4.

Acetyl-L-alanyl-p-nitroanilide, Acetyl-L-leucyl - p-nitroanilide and Formyl-L-methionyl p-nitroanilide were purchased from Sigma Chemicals Co., Poole, Dorset.

5.3 Methods

5.3.1 Maximal Velocity (V_{max}) and Michaelis Constant (K_m)

Determination of Acetyl-L-alanyl p-Nitroanilide with Acetyl Amino-peptidase

The kinetic parameters of this assay reaction were determined using Michaelis-Menten (Ref. 3) mechanics from the equation:-

$$v^* = \frac{V_{max}[S]}{K_m + [S]}$$

where v^* = reaction velocity

[S] = substrate concentration

K_m = Equilibrium or Michaelis-Menten constant

and V_{max} = Maximal Velocity

All values are calculated for a one-substrate enzyme-catalyzed reaction under fixed temperature pH and buffered conditions. The assays were carried out as detailed in section 4.3.2.1 of Chapter 4 (this study).

A range of substrate concentrations were made-up in different samples of Sørensen phosphate buffer (0.1M, pH 8.3) including sodium chloride (0.2 M). The substrate range, 0-2mM, was chosen because this was lower than the substrate concentration (4mM) used in conventional assay and known to represent an excess of Ac-ala-pNA. An aliquot of each sample (500µl) was pre-incubated at 37° prior to the addition of AcAP (20µl, 0.25U). The formation of p-nitroaniline was monitored directly by its absorbance at 405 nm. This assay was carried out for each substrate concentration made, in duplicate and the results (405 nm/min per assay) are shown and analyzed in section 5.4.1).

5.3.2 Variation with pH of the Ac-L-ala-pNA assay

Solutions of the substrate, Ac-L-ala-pNA (4 mM) were made-up in Sørensen's buffer solutions with pHs between 4.8 and 8.9. Aliquots of the substrate solutions (500 µl) were each pre-incubated at 37°C for 5 min, and the assays initiated by the addition of a sample of AcAP (20µl, 0.25U) and the formation of p-nitroaniline was again monitored at 405 nm. The rates at each pH were recorded and the results with pH variation are analyzed in section 5.4.2.

5.3.3 Maximal Velocity (v_{max}) and Michaelis Constant (K_m)

Determination of Acetyl-L-leucyl-pNitroanilide with Acetyl Aminopeptidase

The kinetic parameters were determined as in section 5.3.1. As for section 5.3.1, the assays of AcAP using Ac-L-leucyl-pNA were carried out as follows. Solutions of the substrate (0-1.5 mM) were made-up in Sorenson's phosphate buffer (0.1 M, pH 8.3) including sodium chloride (0.2 M). The addition of AcAP enzyme samples (20 μ l, 0.25 U) initiated the assays (in duplicate) and the rates of formation of p-nitroaniline at 37°C by absorbance monitoring at 405 nm, were recorded and analyzed in section 5.4.3.

5.3.4 Variation with pH of the Acetyl-L-leucyl pNitroanilide

Assay

As for section 5.3.2 a number of Sorenson's phosphate buffer solutions were made-up with pH's ranging between 6.5 and 8.9. Ac-L-leucyl pNA substrate solutions (2 mM) were made-up using each pH buffer and the rate of reaction was determined as before by initiating the reaction, and assay, by the addition of an AcAP sample (20 μ l, 0.25U) to a pre-incubated (37°C, 5 min) substrate sample (500 μ l) and monitoring achieved by absorbance readings at 405 nm. The reaction rates were obtained and analyzed with respect to pH-variation in section 5.4.4.

5.3.5 Maximal Velocity (V_{max}) and Michaelis Constant (K_m)

Determinations of Formyl-L-methionyl-pNA with Acetyl Aminopeptidase

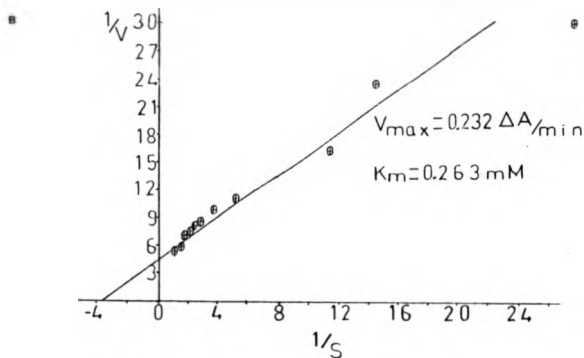
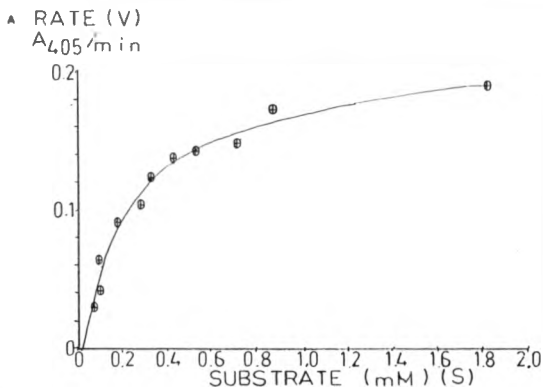
The kinetic parameters were determined as in section 5.3.1. As for section 5.3.1, the assays of AcAP using Formyl-L-methionyl pNA were carried out as follows. Solutions of the substrate F-L-met-pNA (0-3.6 mM) were made up in Sorenson's phosphate buffer (0.1M, pH8.3) including sodium chloride (0.2M) AcAP samples (20 μ l, 0.25U) initiated the reaction and assays, by addition to a sample of each substrate concentration (500 μ l) and the reaction rates monitored at 37°C and 405nm (in duplicate). The rates were obtained and are analyzed in section 5.4.5..

These analyses were designed, not only to compare the specificity of AcAP to each substrate, but also to determine the practical usefulness of each one used. Furthermore, because the same moiety is released in each assay one can begin to draw some conclusions as to the variation that the amino-terminal group (Acetyl or formyl) and the amino-acid derivatized has on the rates of reaction. These conclusions can then be compared to published work concerning AcAP's substrate specificity.

5.4 Results and Discussion

5.4.1 V_{max} and K_m -value Determinations of Acetyl-L-alanyl-p-nitroanilide (AANA) with Acetylaminopeptidase (AcAP)

The kinetics of this reaction were seen to obey Michaelis-Menten first order kinetics observed from the direct rate-concentration plot (Fig. 5.4.1 (a)). As such, the equation quoted on p135 can be used to



A Fig. 5.4.1 (a) Direct Plot of Ac-L-Alanyl-pNA Substrate Concentration vs AcAP Rate

B Fig. 5.4.1 (b) Double Reciprocal Plot to Determine the V_{\max} and K_m values of the Ac-L-Ala pNA Assay of AcAP

A₄₀₅/min

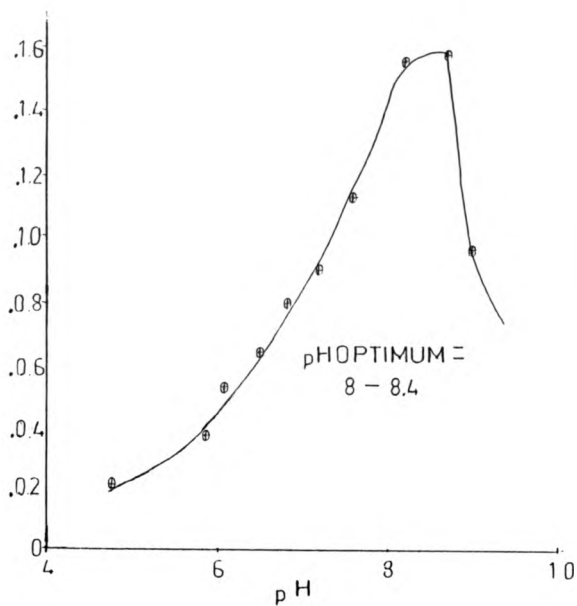


Fig. 5.4.2 pH Profile of the Ac-L-Ala pNA Assay of AcAP

determine the equilibrium constant, K_m and the maximal velocity, V_{max} of this reaction. The K_m -value was calculated as $0.26mM \pm 0.02$ and the V_{max} value as 0.23 ± 0.02 A405 nm/min under the conditions stated.

5.4.2 Variation with pH of the Acetyl-L-alanyl-p-nitroanilide Assay

The pH-profile obtained for this assay following the method outlined in section 5.3.2 shows three things:-

- (1) that there is only one pH-optimum value for this assay.
- (2) that the pH-optimal range of 8.0-8.4 encompasses the pH value of 8.3 used for the assay of AcAP in this study, and
- (3) that the range obtained is in close agreement to that quoted by other workers.

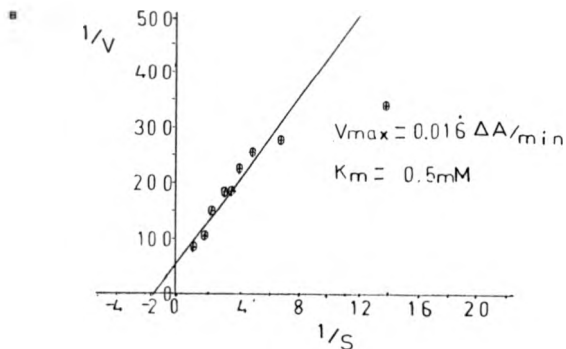
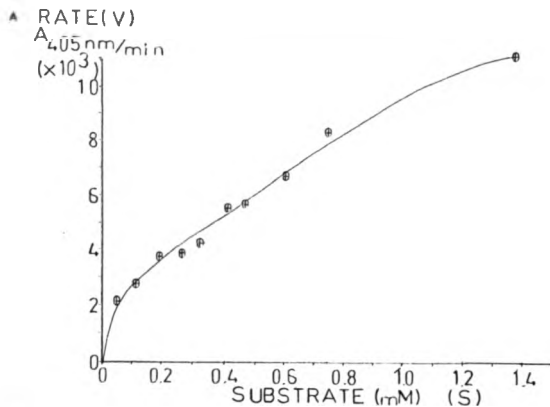
Furthermore, the spontaneous hydrolysis of Acetyl-L-alanyl p-nitroanilide was negligible over the whole pH-range tested.

5.4.3 V_{max} and K_m -value Determinations of Acetyl-L-leucyl-p-nitroanilide with Acetylaminopeptidase

This reaction was shown to follow first order rate Kinetics by the direct plot (Fig. 5.4.3 (a)) and, as such, the Michaelis-Menten equation can be used to calculate the K_m - and V_{max} - values for this reaction.

The K_m -value was calculated to be $0.5 \pm 0.05mM$ and the V_{max} -value to be 0.016 ± 0.004 A405 nm/min. By comparison to those values obtained using Acetyl-L-alanyl p-nitroanilide as substrate one can see that:-

- (a) it requires approximately twice as much substrate to achieve 50%



▲ Fig. 5.4.3 (a) Direct Plot of Ac-L-Leucyl-pNA Substrate Concentration vs AcAP Rate

■ Fig. 5.4.3 (b) Double Reciprocal Plot to Determine the V_{max} and K_m Values of the Ac-L-Leucyl pNA Assay of AcAP

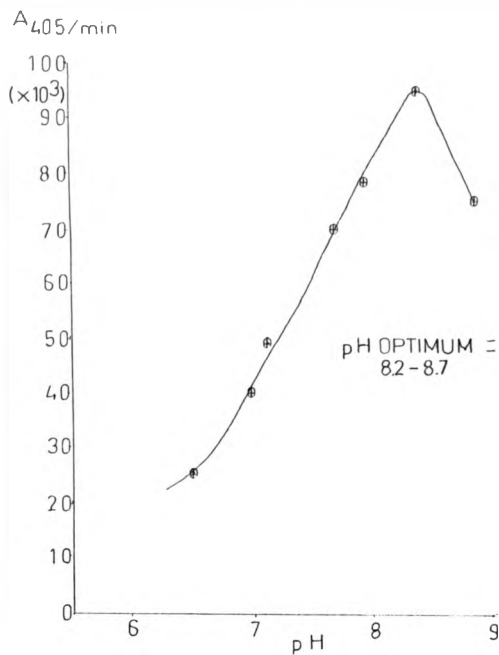


Fig. 5.4.4 pH Profile of the Ac-L-Leucyl pNA Assay of AcAP

of the maximal velocity and

- (b) the maximal velocity achieved was approximately 7% of that observed for Acetyl-L-alanyl p-nitroanilide.

5.4.4 Variation with pH of the Acetyl-L-leucyl-p-nitroanilide with Acetylaminopeptidase

The pH-profile obtained for this assay following the method outlined in section 5.3.4 shows:-

- (1) a similar profile obtained using Acetyl-L-alanyl p-nitroanilide as a substrate,
- (2) a pH-profile optimal range of 8.2-8.7, again similar to that obtained for Acetyl-L-alanyl p-nitroanilide, and,
- (3) that in the pH-range tested, only one pH-maximal value was observed.

Furthermore, spontaneous hydrolysis of Acetyl-L-leucyl p-nitroanilide was found to be negligible over the whole pH-range tested and under the conditions of the assay.

5.4.5 V_{max} and K_m Value Determinations of Formyl-L-methionyl p-nitroanilide with Acetylaminopeptidase

The kinetics of this reaction were seen to obey Michaelis-Menten mechanics observed from the direct plot of enzymic-rate against substrate concentration (Fig 5.4.5 (a)). As for Acetyl-L-alanyl p-nitroanilide, and Acetyl-L-leucyl p-nitroanilide the kinetic constants were calculated from a double reciprocal plot (Fig 5.4.5 (b)) and were calculated to be $1.33 \pm 0.02\text{mM}$ for the K_m -value and 0.125 ± 0.02 A405nm for the V_{max} -value.

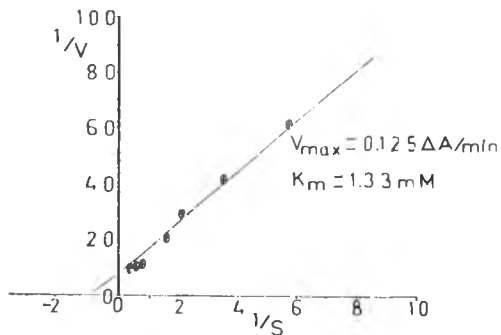
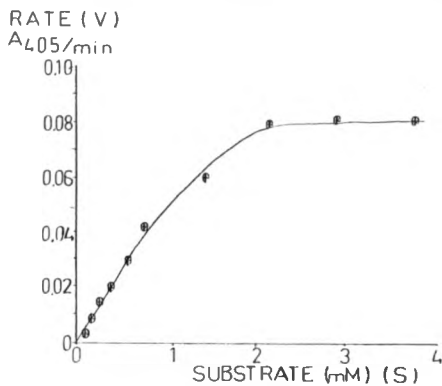


Fig. 5.4.5 (a) Direct Plot of F-L-Methionyl-pNA Substrate Concentration vs AcAP Rate

Fig. 5.4.5 (b) Double Reciprocal Plot to Determine the V_{max} and K_m Values of the F-L-Methionyl pNA Assay of AcAP

Comparison of these values to those obtained for Acetyl-L-alanyl p-nitroanilide and Acetyl-L-leucyl p-nitroanilide shows that the maximal rate is 55% and 750% of the values obtained for the former substrates respectively, but yet, to obtain the half-maximal rate (or concentration of substrate at which the reaction rate is half that of the maximum possible, equivalent to the K_m -value) one sees that one needs approximately five times and three times the amount required for the same effect in the Acetyl-L-alanyl and Acetyl-L-leucyl p-nitroanilide assays respectively.

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